

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

In re Patent Application of

MASSFELDER et al.

Atty. Ref.: 3665-133

Serial No. 10/520,085

TC/A.U.: 1643

Filed: January 5, 2005

Examiner: Gussow

For: USE OF PTHRP ANTAGONISTS FOR TREATING RENAL CELL
CARCINOMA

August 28, 2009

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Applicant hereby appeals the final rejection of claims 17-20, 23-26, 31 and 33-35, in the Office Action dated February 23, 2009, and submits the present Appeal Brief pursuant to 37 CFR § 41.37.

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

Table of Contents	Page
(1) REAL PARTY IN INTEREST	3
(2) RELATED APPEALS AND INTERFERENCES	4
(3) STATUS OF THE CLAIMS	5
(4) STATUS OF THE AMENDMENTS	6
(5) SUMMARY OF CLAIMED SUBJECT MATTER	7
(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL	9
(7) ARGUMENT	10
(8) CLAIMS APPENDIX	30
(9) EVIDENCE APPENDIX	32
(10) RELATED PROCEEDINGS APPENDIX	36

(1) REAL PARTY IN INTEREST

The following are the real parties in interest:

UNIVERSITE LOUIS PASTEUR, 4, RUE BLAISE-PASCAL, STRASBOURG,
FRANCE 67000;

INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE
(INSERM), 101, RUE DE TOLBIAC, PARIS CEDEX 13, FRANCE 75654; and
HOPITAUX UNIVERSITAIRES DE STRASBOURG, 1, PLACE DE
L'HOPITAL, B.P. 426, STRASBOURG CEDEX, FRANCE 67091,

by way of an Assignment from the appellants, recorded in the U.S. Patent and
Trademark Office on April 18, 2005, at Reel 016935, Frame 0676.

(2) RELATED APPEALS AND INTERFERENCES

The appellant, the appellant's legal representative, and the assignees are not aware of any related prior or pending appeals or interferences or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(3) STATUS OF THE CLAIMS

Independent claim 17, and dependent claims 18-20, 23-26, 31 and 33-35, are pending.

Independent claim 17, and dependent claims 18-20, 23-26, 31 and 33-35, have been finally rejected.

The application was filed with claims 1-16, which were canceled by way of a Supplemental Amendment filed April 18, 2005, which added new claims 17-37.

Claims 17, 33, 34 and 35, were amended, and claims 21, 22, 37-30, 32, 36 and 37, canceled, without prejudice, in an Amendment filed April 16, 2007. Claim 17 was further revised, without prejudice, in an Amendment filed September 6, 2007. Claim 17 was further revised, and new claims 38-52 added, without prejudice, in an Amendment After Final Rejection filed January 10, 2008.

Claim 17 was revised and claims 38-52 canceled, without prejudice, in an Amendment filed April 22, 2008. Claim 17 was again revised, without prejudice, in an Amendment filed November 27, 2008.

Independent claim 17, and dependent claims 18-20, 23-26, 31 and 33-35, have been rejected in the Office Action of February 23, 2009 and are the subject of the present appeal.

A copy of all the rejected claims 17-20, 23-26, 31 and 33-35, i.e., the claims involved in the appeal, is attached as a Claims Appendix, pursuant to Rule 41.37(c)(1)(viii).

(4) STATUS OF THE AMENDMENTS

No amendments have been filed subsequent or in response to the Final Rejection of February 23, 2009.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Pursuant to 37 CFR § 41.37(a)(2)(v), the following is a concise explanation of the subject matter defined in each of the independent claims involved in the appeal, which shall refer to the specification by page and line number, and to the drawing, if any, by reference characters.

Claim 17 is the only independent claim of the claims on appeal.

Independent claim 17 defines a method for treating a kidney cancer (see for example, independent claim 17, page 6, lines 5-12 and 18-19 of the specification as well as originally-filed claim 1) which involves the administration to a subject of an effective dose of a PTHrP antagonist (see for example, independent claim 17, page 6, lines 9-10 of the specification as well as originally-filed claim 1) for inhibiting or decreasing a tumor growth (see for example, independent claim 17, page 6, lines 13-17 of the specification as well as originally-filed claim 3) or a pharmaceutical composition containing it (see for example, independent claim 17, page 6, line 32 through page 7, line 2 of the specification), said PTHrP antagonist being an anti-PTHrP antibody (see for example, independent claim 17, page 7, lines 7-10 and 30-33 of the specification as well as originally-filed claims 5 and 12) that binds amino acids 34-53 of PTHrP (see for example, independent claim 17, page 9, lines 10-15 of the specification).

The details of dependent claim 18 finds support, for example, at lines 5-8 on page 6 of the specification.

The details of dependent claim 19 find support, for example, on page 6, lines 9-12 of the specification.

The details of dependent claim 20 find support, for example, on page 6, lines 11-12 of the specification.

The details of dependent claim 23 find support, for example, on page 6, lines 27-29 of the specification.

The details of dependent claim 24 find support, for example, on page 7, lines 7-8 of the specification.

The details of dependent claim 25 find support, for example, on page 7, lines 9-10 of the specification.

The details of dependent claim 26 find support, for example, on page 7, lines 9-10 of the specification.

The details of dependent claim 31 find support, for example, on page 7, lines 30-32 of the specification.

The details of dependent claim 33 find support, for example, on page 7, lines 33-34 of the specification.

The details of dependent claim 34 find support, for example, on page 8, lines 26-30 of the specification.

The details of dependent claim 35 find support, for example, on page 8, lines 30-31 of the specification.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following ground of rejection is presented for review:

Whether the invention of claims 17-20, 23-26, 31 and 33-35, are supported by an adequate written description, as required by 35 U.S.C. § 112, first paragraph.

(7) ARGUMENT

The claimed invention is adequately described by the present specification, and the rejection of claims 17-20, 23-26, 31 and 33-35 under 35 U.S.C. § 112, first paragraph, should be reversed. Consideration of the following and attached in this regard is requested.

The Examiner has asserted, as the basis for the rejection, that the "genus" of antibodies included in the method of independent claim 17 (i.e., "an anti-PTHrP antibody that binds amino acids 34-53 of PTHrP" (emphasis added)) are allegedly not described in the specification.

One of ordinary skill in the art will appreciate from a review of the specification, as well as the advanced level of skill in the art and the evidence of record, that the applicants were in possession of the claimed invention at the time the application was filed.

The specification describes anti-PTHrP (34-53) antibodies, as required by the claimed invention.

One of ordinary skill will appreciate that a description of an "anti-PTHrP (34-53) antibody" refers generally to an antibody that binds residues 34-53 of PTHrP. The following evidence is submitted in support of the general nature of such a disclosure and the general understanding of one of ordinary skill in the art:

(a) Okada et al "Immunohistochemical Localization of Parathyroid Hormone-related Protein in Canine Mammary Tumors" Vet Pathol **34**: 356-359 (1997) (copy attached as Evidence Appendix (a)) (describing an antibody to "PTHrP (1-36)" (page

356 right column), the use of a "commercially available rabbit-derived anti-PTHrP (34-53) antibody" (id.), and the N-terminus (1-36) and midregion (36-111) of PTHrP);

(b) Verheijen et al, "Parathyroid hormone-related peptide (PTHrP) induces parietal endoderm formation exclusively via the Type I PTH//PTHrP receptor" *Mechanisms of Development* 81 (1999) 151-161 (copy attached as Evidence Appendix (b)) (describing the N-terminus of PTHrP as "PTHrP (1-34)" (see page 151, left column), the use of the N-terminal fragment "PTHrP(1-34)" and full length version "PTHrP(1-141)" (see page 152, right column), and fragments spanning amino acids 67-86, 67-94 and 107-139 as "PTHrP(67-86)", "PTHrP(67-94)" and "PTHrP(107-139)", respectively (see page 153, left column and Figure 1, and the "Materials" section on page 158 which describes the source of peptides and antibodies));

(c) Thorikay et al., "Synthesis of a gene encoding parathyroid hormone-like protein-(1-141): purification and biological characterization of the expressed protein" *Endocrinology*, Vol 124, 111-118 (1989) (abstract) (copy attached as Evidence Appendix (c)) (describing "PTHLP" as a 141 amino acid protein designated "PTHLP-(1-141)");

(d) Fenton et al., "A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts." *Endocrinology*. 1991 Oct;129(4):1762-8 (Abstract) (copy attached as Evidence Appendix (d)) (describing a carboxy fragment of PTHrP as "PTHrP-(107-139)");

(e) Santos et al "Up-regulation of parathyroid hormone-related protein in folic acid-induced acute renal failure" *Kidney International*, vol. 60 (2001), pp 982-995

(copy attached as Evidence Appendix (e)) (describing "anti-PTHrP antibody Ab-2 (Oncogene, Uniondale, NY, USA), [as] recognizing the sequence 34 to 53 of human and rat PTHrP" on page 983);

(f) Garcia-Ocana et al "Cyclosporine increases renal parathyroid hormone-related protein expression in vivo in the rat" Transplantation, vol 65, 860-863, No. 6, March 27, 1998 (copy attached as Evidence Appendix (f)) (describing "anti-PTHrP antibody Ab-2 (Oncogene, Uniondale, NY), [as] recognizing the sequence (34-53) of human and rat PTHrP" on page 861); and

(g) Richard, et al. "Humoral Hypercalcemia of Malignancy, Severe Combined Immunodeficient/Beige Mouse Model of Adult T-Cell Lymphoma Independent of Human T-Cell Lymphotropic Virus Type-1 Tax Expression" Am J Pathol. 2001 June; 158(6): 2219-2228 (copy attached as Evidence Appendix (g)) (describing "polyclonal rabbit anti-PTHrP (PTHrP amino acids 34 to 53) (1:100, Ab-2, Oncogene Research Products, Cambridge, MA)").

Each of the above-noted references describes fragments of PTHrP by the amino acid positions, in parentheses, in a manner similar to the applicants disclosure and claims.

The following further references, for example, similarly describe fragments of PTHrP:

(h) Burton et al., "Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma" 1990, Biochemical and

Biophysical Research Communications, Vol. 167, No. 3, pages 1134-1138 (copy attached as Evidence Appendix (h));

(i) Ogata et al (EP1197225) (copy attached as Evidence Appendix (i));

(j) Hoare et al "Specificity and stability of a new PTH1 receptor antagonist, mouse TIP(7-39)" Peptides, 2002, vol 23, No. 5, pp 989-998 (copy attached as Evidence Appendix (j)); and

(k) Sato et al (U.S. Patent No. 6,903,194) (copy attached as Evidence Appendix (k)).

Sato et al describes "Humanized anti-PTHrP (1-34) Antibody" in Figures 13 and 14. Moreover, Sato et al describes the use of a fragment "[PTHrP(1-34)]" as an antigen to produce antibodies as follows:

"PTHrP used for the immunization of animals includes peptides having the whole or part of the amino acid sequence of PTHrP prepared by recombinant DNA technology or chemical synthesis, and PTHrP derived from supernatants of cancer cells causing hypercalcemia. For example, a peptide [PTHrP(1-34)] comprising the 1st to 34th amino acids of the known PTHrP (Kemp, B. E. et al., Science (1987) 238, 1568-1570) may be used as the antigen." See column 7, lines 48-55 of Sato.

Sato et al exemplifies therefore the general understanding of one of ordinary skill in the art that an "anti-PTHrP (1-34) antibody" is a general description of an antibody which binds to the PTHrP (1-34) fragment. The general understanding is not specific to the fragment of amino acids 1-34 of PTHrP but will be understood to be generally applicable to a description of other enumerated fragments.

Further, Sato describes antibodies binding human PTHrP as "anti-human PTHrP antibodies" and generally antibodies which bind PTHrP as "Anti-PTHrP Antibody". See column 10, last line, column 22, line 56, and, for example, column 23, lines 25 and 37-38 of Sato.

One of ordinary skill in the art will appreciate that "an anti-PTHrP (34-53) antibody" is a general recitation and description of an antibody which binds to the fragment of PTHrP spanning amino acids 34-53.

The present specification describes the following as examples of an anti-PTHrP antibody which may be an antagonist according the disclosed invention: the anti-PTHrP(1-34) antibodies (human, rat) of Bachem (Bachem Biochimie Sarl, Voisins-le-Bretonneux, France), the anti-PTHrP(34-53) antibody (Ab-2, human) of Oncogene (France Biochem, Meudon, France), the antibody #23-57-137-1 (described in particular in the patent application EP1197225) and the anti-PTHrP(107-139) antibody (human) obtained by conventional methods of antibody preparation. See page 9, lines 10-15 of the present specification. One of ordinary skill in the art will further appreciate from, for example, page 21, line 31 ("anti-PTHrP (34-53)"), page 17, lines 25-28 ("The anti-PTHrP(34-53) antibody (Ab-2, human) was obtained from Oncogene (France Biochem, Meudonm France) and the anti-PTHrP(107-139) antibody (human) was a gift of Dr. P. Esbrit (Fundacion, Jimenez Diaz, Madrid, Spain)") and page 27, line 1 ("Int. region: anti-PTHrP (34-53) antibody (Ab-2, Oncogene) 2 µg/ml") of the specification, that the present specification describes the use of anti-PTHrP antibodies from a number of sources as

exemplifications of anti-PTHrP antibodies which bind to the amino acid fragment described numerically in parentheses (i.e., fragments of PTHrP spanning amino acids 34-53 and 107-139 in the above-noted passages).

The claims on appeal relate to an anti-PTHrP antibody binding the specified fragment of the claims. The specification describes a number of PTHrP antagonists useful in the disclosed invention.

The Examiner is of the view that the present disclosure provides a written description for a "genus" of anti-PTHrP antibodies and a specific species of the "subgenus" of the anti-PTHrP antibodies of the claims, without describing the subgenus of the claims. The Examiner is further understood to believe that the specification only describes a commercially available species of the claimed "subgenus", i.e., Ab-2 from Oncogene (which is believed to now be available from CALBIOCHEM EMD Chemicals under the name "Anti-PTHLP (Ab-2) (34-53) Rabbit pAb").

The Examiner is believed to have agreed during the interview of October 28, 2008, that the specification teaches a peptide fragment of PTHrP spanning amino acids 34-53 as an antigen to which an anti-PTHrP antibody can be made to bind.¹ The Examiner is believed to have agreed during the interview that an antibody which binds to a fragment of PTHrP spanning amino acids 34-53 would be reasonably

¹ See page 9 of the Remarks of the Amendment filed November 27, 2008, subsequent to the Examiner Interview, wherein a similar summary was provided. This summary of the Examiner

referred to as an anti-PTHrP (34-53) antibody.² The Examiner is believed to have agreed during the interview that the specification, taken with the generally advanced level of skill in the art, teaches one of ordinary skill in the art how to make and use an anti-PTHrP (34-53) antibody according to the claimed invention.³

The applicants have presented evidence in the form of a Declaration of MASSFELDER executed February 14, 2008 in this regard (copy attached as Evidence Appendix (I)). More specifically, the Declaration of MASSFELDER executed February 14, 2008, describes production of two further distinct monoclonal anti-PTHrP (34-53) antibodies, called PTH1 H413 and PTH2 E1131 which demonstrated *in vivo* antitumoral effect, as was demonstrated with the exemplified antibody in Figures 4, 6 and 8 of the specification.

The Examiner believes however that the specification fails to describe the claimed method wherein the PTHrP antagonist is an anti-PTHrP (34-53) antibody.

Antagonists are further described in the specification as including a compound, such as an anti-PTHrP antibody, which inhibits the binding of a ligand, such as PTHrP, to a PTHrP receptor. Examples of anti-PTHrP antibodies include antibodies such as a humanised antibody, a human antibody, a chimeric antibody, an antibody (such as the antibody #23-57-137-1 (which binds PTHrP(1-35) obtained from a hybridoma (such as the hybridoma #23-57-137-1) or a fragment of an anti-PTHrP which inhibits binding of a ligand to the receptor and/or a modified form of

Interview by the appellant has not been corrected by the Examiner in subsequent actions in the record.

² See fn. 1.

such a fragment. The antibody can be polyclonal or monoclonal. See page 8, lines 23-31 and page 9, lines 4-9 of the specification.

The present specification describes the following as examples of an anti-PTHrP antibody which may be an antagonist according the disclosed invention: the anti-PTHrP(1-34) antibodies (human, rat) of Bachem (Bachem Biochimie Sarl, Voisins-le-Bretonneux, France), the anti-PTHrP(34-53) antibody (Ab-2, human) of Oncogene (France Biochem, Meudon, France), the antibody #23-57-137-1 (described in particular in the patent application EP1197225) and the anti-PTHrP(107-139) antibody (human) obtained by conventional methods of antibody preparation. See page 9, lines 10-15 of the present specification.

The specification further describes the use of fragments of PTHrP as antigens to produce anti-PTHrP antibodies in mammals, such as a rodent (e.g., mouse, rat or hamster), a rabbit or a monkey. See page 9, line 29 through page 10, line 4 of the specification. Production of anti-PTHrP antibodies from human cells is also described. See page 11, lines 16-26 of the specification. Recombinant anti-PTHrP antibodies are described. See page 11, line 27 through page 12, line 2 of the specification.

Page 14, lines 12-14 of the specification describes the results of Figure 4 of the specification as showing the "effect of the antibodies against the various regions of PTHrP on the proliferation of the tumor cells 786-0 *in vitro* measured by the

³ See fn. 1.

number of cells... ". As noted in the following reproduction (emphasis added), Figure 4 describes these "regions" as "N-term", "Region int" and "C-term".

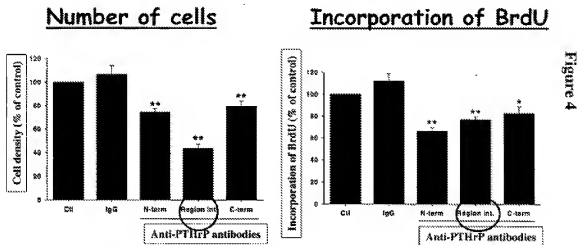


Figure 4, as well as Figures 6 and 8, of the specification describe the effect of binding to regions, as opposed to the binding of specifically exemplified antibodies.

One of ordinary skill in the art would not interpret Figure 4 of the specification, or any other aspect of the specification, to describe only a specific effect of a commercially available antibody used by the applicants to exemplified their claimed invention. One of ordinary skill in the art would not believe from a review of the specification that the applicants invention requires the use of only the commercially available antibody used by the applicants to exemplify their disclosed invention.

The specification further defines the regions of Figure 4 as follows (see page 26, line 33 through page 27, line 2 of the specification):

"N-term: anti-PTHrP(1-34) antibody (Bachem) 1.5 µg/ml

Int. region: anti-PTHrP (34-53) antibody (Ab-2, Oncogene)
2 µg/ml
C-term: anti-PTHrP(107-139) antibody (P. Esbrit, Madrid,
Espagne) 5 µg/ml” .

One of ordinary skill in the art will appreciate that Figure 4 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants invention.

Figure 6 of the specification similarly describes results relating to anti-PTHrP antibodies which bind to regions “N-term” (i.e., PTHrP(1-34)), “Region Int.” (i.e. PTHrP (34-53)), and “C-term” (i.e., PTHrP(107-139)) of PTHrP. The corresponding description of the specification (i.e., page 27, lines 14-23) describes the general applicability of the results of Figure 6 as representing

“the effect of the antibodies directed against the different regions of PTHrP on the proliferation of the UOK-126 tumor cells *in vitro* ...” (emphasis added).

One of ordinary skill in the art will appreciate that Figure 6 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants invention.

Figure 8 of the specification similarly describes results relating to anti-PTHrP antibodies which bind to regions “N-term” (i.e., PTHrP(1-34)), “Region Int.” (i.e. PTHrP (34-53)), and “C-term” (i.e., PTHrP(107-139)) of PTHrP. The corresponding

description of the specification (i.e., page 28, lines 1-12) describes the general applicability of the results of Figure 8 as representing

“the effect of the antibodies directed against the different regions of PTHrP on the proliferation of the UOK-128 tumor cells *in vitro* ...” (emphasis added).

One of ordinary skill in the art will appreciate that Figure 8 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants invention.

The applicants submit that one of ordinary skill in the art will appreciate, from the whole of the specification, that the applicants were in possession of the claimed invention, relating to the use of an anti-PTHrP (34-53) antibody. The specification is not limited to the specific Ab-2 anti-PTHrP (34-53) antibody used in the examples.

The Examiner relies on In re Smith 173 USPQ 679 (CCPA 1972) (copy attached as Evidence Appendix (m)) for the assertion that

“It cannot be said that a subgenus is necessarily described by a genus encompassing it and a species upon which it reads.” See page 4 of the Office Action dated August 1, 2008.

The Examiner’s reliance on In re Smith is misplaced. Consideration of the following in this regard is requested.

The claims under consideration in In re Smith included the following recitation of an organic compound for treating the surface of a pigment to be used in an emulsion coating composition:

"said organic compound being a monomeric organic compound characterized by at least one non-polar organic hydrophobic group containing at least 8 carbon atoms in a hydrocarbon structure, which group in the form of its monocarboxylic acid" see 173 USPQ 680.

The issue in In re Smith was whether an earlier-filed application provided written description support to antedate a reference. The Smith court summarized from the findings of the Board below which found the earlier filed application failed to

"mention ... the requirement that the coating compound must be a monomer having at least 8 carbon atoms in its hydrophobic moiety, and that more than one polar group was contemplated, all of which is recited in claim 1." See 173 USPQ 682.

The Board rejected the appellants assertions that the following generic disclosure supported the claimed recitation:

"The treatment of pigments with polar agents is not new per se and can be accomplished by several methods employing a variety of effective compounds. In general these methods involve surface coating the pigment with an oil soluble polar organic compound. Among the polar organic compounds are acidic resins, water soluble resins, water insoluble metallic resins, long chain fatty acids, their salts and soaps, benzene carboxylic acid and its salts, naphthenic acids and their soaps and salts, cationic active agents, e.g., alkyl amine salts and quaternary ammonium compounds containing at least 12 carbon atoms in an alkyl group or groups, e.g., lauryl pyridinium bromide, and long chain (at least 12 carbon atoms) fatty acid-containing organic Werner complexes."

Id.

The appellants unsuccessfully argued that

"It is obvious that the surface coating organic compounds recited in the foregoing paragraph are monomeric, have a hydrocarbon structure of at least 8 carbon atoms, except for benzene carboxylic acid which contains six carbon atoms in a hydrocarbon group, and contain at least one carboxy or carboxylate group. If appellant's claims had been drawn more broadly, they would be supported by the parent application. They can be described as subgeneric claims because they delineate the invention more specifically by reciting that the organic material used to coat the pigment is monomeric, contains at least 8 carbon atoms and at least one carboxy or carboxylate group." Id.

The disclosure considered in In re Smith therefore did not contain any written description support for the claimed compounds of "at least 8 carbon atoms" of the claims. Rather, the disclosure considered in In re Smith contained a disclosure of "at least 12 carbon atoms" which the appellants argued supported the lesser inclusive range of the claimed. In affirming the Board, the Smith court likened the facts of Smith to those considered in In re Ahlbrecht (168 USPQ 293 (CCPA 1971) (copy attached as Evidence Appendix (n)) wherein a disclosure of fluorinated esters having 2-12 CH₂ groups failed to provide written description support for a claim to fluorinated esters having 3-12 CH₂ groups. See 173 USPQ 684. In deciding Smith, the court rejected the "rule" of In re Risse (154 USPQ 1 (CCPA 1967) (copy attached as Evidence Appendix (o))

"to the effect that the disclosure of a genus and a species of a subgenus is a sufficient description of the subgenus. We do not now feel that such a rule is consonant with

either the letter or spirit of the description requirement of § 112." See 173 USPQ 683.

Unlike the disclosure considered in In re Smith, the present disclosure provides an adequate written description of the general anti-PTHrP (34-53) antibody of the claims, as detailed above. The present facts do not require consideration of whether claims to a range within a broad general disclosure of the specification but outside a less general disclosure or specific disclosure of the specification are supported by the specification, as was the case with In re Smith and In re Ahlbrecht. The present disclosure describes the claimed invention and uses a commercially available product to exemplify the disclosed and claimed invention. One of ordinary skill in the art will appreciate that the applicants described their invention to include use of an anti-PTHrP (34-53) antibody in the claimed method.

As explained in Capon v. Eshhar, 76 USPQ.2d 1078, 1084 (Fed. Cir. 2005) (copy attached as Evidence Appendix (p)),

The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. See *Enzo Biochem*, 296 F.3d at 1330 (the written description requirement "is the quid pro quo of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reiffin v. Microsoft Corp.*, 214 F.3d 1342, 1345-46 (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker*, 559 F.2d 588, 592 n.4 (C.C.P.A. 1977) (the goal of the written description requirement

is “to clearly convey the information that an applicant has invented the subject matter which is claimed”). The written description requirement thus satisfies the policy premises of the law, whereby the inventor’s technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

Further, the Court of Appeals for the Federal Circuit has explained as follows in In re Kenneth Alonso (2008-1079 Fed. Cir. October 30, 2008)⁴ (copy attached as Evidence Appendix (q)) with regard to the written description requirement in the case of a criteria for a claim defining a genus of antibodies.

The written description requirement of 35 U.S.C. § 112, ¶ 1, is straightforward: “The specification shall contain a written description of the invention” To satisfy this requirement, the specification must describe the invention in sufficient detail so “that one skilled in the art can clearly conclude that the inventor invented the claimed invention as of the filing date sought.

The requirement “serves a teaching function, as a ‘*quid pro quo*’ in which the public is given ‘meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time.’”

The requirement is rigorous, but not exhaustive: “[I]t is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a

⁴ <http://caselaw.lp.findlaw.com/data2/circs/fed/081079p.pdf> (April 7, 2009), page 5 (citations omitted).

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

person of skill in the art that the inventor possessed the invention. *LizardTech*, 424 F.3d at 1345.

The applicants submit that the determination of what is needed to support generic claims to biological subject matter depends on a variety of factors, such as the existing knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter.⁵ For example, it is unnecessary for the specification to provide a description of proteins which are already known in the prior art.⁶

In *Enzo Biochem v. Gen-Probe, Inc.*, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002) (copy attached as Evidence Appendix (r)), the court stated that the written description requirement would be met for all of the claims of the patent at issue if the functional characteristic of the claimed invention was coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed.

Finally, the applicants note that the Federal Circuit has stated that as long as an applicant has disclosed a “fully characterized antigen,” either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository, the applicant can then claim an antibody by its binding affinity to that described antigen.⁷

⁵ See *Capon* 76 USPQ.2d 1085.

⁶ See *Capon* 76 USPQ.2d 1087.

⁷ See *Noelle v. Lederman*, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004) (copy attached as Evidence Appendix (s))

In the present application, the polypeptide sequence of PTHrP is known and fully characterized⁸ and the specification teaches that antibodies can be made to fragments of the PTHrP polypeptide⁹. As noted previously, antibodies that bound to known PTHrP fragments (e.g., the fragment spanning amino acid residues 34-53) were known in the art and commercially available¹⁰, demonstrating that both the antigen and antibodies that bound thereto were fully characterized by structure and formula.

The applicants have demonstrated the use of anti-PTHrP (34-53) antibodies in the treatment of kidney cancer by using in the example the anti-PTHrP (34-53) antibody of Oncogene. However, the teaching is not limited to this specific antibody. One of ordinary skill in the art deduces for these results that any anti-PTHrP (34-53) antibody will have the same therapeutic effect. The applicants have provided a Declaration of Dr. Thierry MASSFELDER executed February 14, 2008 (copy attached as Evidence Appendix (I)) with results obtained with two other anti-PTHrP (34-53) antibodies prepared by the applicants and having the therapeutic effect.

In addition, the specification teaches how to make and use anti-PTHrP (34-53) antibodies, as claimed, as previously acknowledged by the Examiner.¹¹

The specification teaches the use of an anti-PTHrP antibody. The PTHrP targeted-region 34-53 only contains 20 amino acids. Therefore, it is a well-defined

⁸ See for example, page 9, lines 22-28 of the present specification.

⁹ See page 9, lines 29-33 of the present specification, for example.

¹⁰ See additional Calbiochem product sheet as well as the references cited therein (copy attached as Evidence Appendix (I)).

¹¹ See for example, pages 9-10 of the Amendment filed November 27, 2008.

antigen.¹² Accordingly, the antibodies would have been expected to not vary substantially within the subgenus of the claims. The exemplified anti-PTHrP (34-53) antibody Ab-2 (Oncogene) is sufficiently representative of the subgenus.

As explained by the Federal Circuit in In re Kenneth Alonso¹³

A genus can be described by disclosing: (1) a representative number of species in that genus; or (2) its "relevant identifying characteristics," such as "complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics."

In the present specification, the amino acids bound by the antibody are disclosed: region 34-53 of PTHrP. The structure of the antigen, and hence the antibody of the claims, is reasonably predictable.

The Declaration Dr. Thierry MASSFELDER executed February 14, 2008 (copy attached as Evidence Appendix (I)) demonstrates the predictability of the subgenus of the claims as well as the insubstantial variation.

The present specification describes, for example, that the antibodies used as exemplified embodiments of the claimed invention bind to an intermediate region of

¹² The following examples of the antigen has been previously described in the Remarks of the Response filed April 13, 2009:

Homo sapiens (human) (NP_945316.1*)	34 AEIRATSEVSPNSKPSNTK 53
Pan troglodytes (chimpanzee) (XP_001142021.1**)	34 AEIRATSEVSPNSKPSNTK 53
Canis familiaris (dog) (NP_001003303.1*)	34 AEIRATSEVSPNSKPAINTK 53
Bos taurus (cow) (NP_777178.1*)	34 AEIRATSEVSPNSKPAINTK 53
Mus musculus (mouse) (NP_032996.1*)	34 AEIRATSEVSPNSKPAINTK 53
Rattus norvegicus (rat) (NP_036768.1*)	34 AEIRATSEVSPNSKPAINTK 53

* <http://sib.uniprot.org/>

** <http://www.ebi.ac.uk/>

¹³ <http://caselaw.lp.findlaw.com/data2/circs/fed/081079p.pdf> (April 7, 2009), page 6 (citations omitted).

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

PTHrP which targets amino acids 34-53¹⁴. Contrary to the Examiner's assertion¹⁵, one of ordinary skill will appreciate that the exemplified antibody Ab-2 (Oncogene) binds to amino acids 34-53 of PTHrP.¹⁶

One of ordinary skill in the art will appreciate from the present specification as well as from the generally advanced level of skill in the art that the exemplified antibody Ab-2 (Oncogene) binds to amino acids 34-53 of PTHrP.¹⁷ The PTHrP-targeted region of the claims is described generally and through an exemplified embodiment in the present specification.

The appellants respectfully submit that the claims are supported by an adequate written description. One of ordinary skill in the art will appreciate that the appellants were in possession of the claimed invention at the time the application was filed.

Reversal of the Section 112, first paragraph "written description", rejection is requested.

¹⁴ See for example, page 27, line 1 of the specification ("Int. region: anti-PTHrP (34-53) antibody (Ab-2, Oncogene) 2 µg/ml); ¶8. of the Rule 132 DECLARATION of Dr. OULAD ABDELGHANI executed November 27, 2008 (copy attached as Evidence Appendix (u)); as compared to the Examiner's suggestion that that the antibodies of the examples bind to an intermediate region of amino acids 36-53 ("in the examples the antibodies used for the amino terminal is 1-34, the intermediate is 36-53, and the carboxy terminal is 107-139 (pages 26-27)."^{see} page 4, lines 2-3 of the Office Action dated February 23, 2009).

¹⁵ "While one would understand that the antibody from Oncogene binds to an epitope within residues 36-53, there is no disclosure of residues 34-53, except provided in the name of the Oncogene antibody." See page 4, lines 9-11 of the Office Action dated February 23, 2009.

¹⁶ See for example, ¶¶8., 15. and 17.-23. of the Rule 132 DECLARATION of Dr. OULAD ABDELGHANI executed November 27, 2008 (copy attached as Evidence Appendix (u)), and the passages of the specification referred to therein.

¹⁷ See for example, ¶¶4.-8. of the Rule 132 DECLARATION of Dr. OULAD ABDELGHANI executed November 27, 2008 (copy attached as Evidence Appendix (u)), and the passages of the specification and references referred to therein.

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

The claims are submitted to be in condition for allowance and Reversal of the final rejection is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: /B. J. Sadoff/
B. J. Sadoff
Reg. No. 36,663

BJS:
901 North Glebe Road, 11th Floor
Arlington, VA 22203
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

(8) CLAIMS APPENDIX

17. A method for treating a kidney cancer comprising the administration to a subject of an effective dose of a PTHrP antagonist for inhibiting or decreasing a tumor growth or a pharmaceutical composition containing it, said PTHrP antagonist being an anti-PTHrP antibody that binds amino acids 34-53 of PTHrP.

18. Method according to claim 17, wherein said subject is a human subject.

19. Method according to claim 17, wherein said kidney cancer is selected from the group consisting of papillary carcinoma (chromophiles), chromophobe cell carcinoma, Bellini carcinoma and unclassified renal cell carcinomas.

20. Method according to claim 19, wherein said kidney cancer is clear cell carcinoma (CCC).

23. Method according to claim 17, wherein the kidney cancer is a solid malignant tumour.

24. Method according to claim 17, wherein the PTHrP antagonist is a compound binding the PTHrP receptor and inhibiting partially or totally a binding of PTHrP to its receptor.

25. Method according to claim 24, wherein the PTHrP antagonist is a PTHrP receptor antagonist.

26. Method according to claim 25, wherein the PTHrP antagonist is a PTHrP competitive antagonist.

31. Method according to claim 17, wherein the PTHrP antagonist is a compound binding a ligand of the PTHrP receptor, and inhibiting partially or totally a binding of PTHrP to its receptor.

33. Method according to claim 17, wherein the PTHrP antagonist is a humanised anti-PTHrP antibody.

34. Method according to claim 17, wherein the anti-PTHrP antibody is selected from a humanised antibody, a human antibody, a chimeric antibody, an antibody obtained from a hybridoma and a fragment thereof and a modified form of said fragment.

35. Method according to claim 17, wherein the anti-PTHrP antibody is a polyclonal or monoclonal antibody.

(9) EVIDENCE APPENDIX

Attached:

(a) Okada et al "Immunohistochemical Localization of Parathyroid Hormone-related Protein in Canine Mammary Tumors" Vet Pathol **34**: 356-359 (1997) (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW).

(b) Verheijen et al, "Parathyroid hormone-related peptide (PTHrP) induces parietal endoderm formation exclusively via the Type I PTHrP receptor" Mechanisms of Development **81** (1999) 151-161 (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW).

(c) Thorikay et al., "Synthesis of a gene encoding parathyroid hormone-like protein-(1-141): purification and biological characterization of the expressed protein" Endocrinology, Vol **124**, 111-118 (1989) (abstract) (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW).

(d) Fenton et al., "A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts." Endocrinology. 1991 Oct;129(4):1762-8 (Abstract) (submitted November 27, 2008 and acknowledged to

have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW).

(e) Santos et al "Up-regulation of parathyroid hormone-related protein in folic acid-induced acute renal failure" *Kidney International*, vol. 60 (2001), pp 982-995 (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW) .

(f) Garcia-Ocana et al "Cyclosporine increases renal parathyroid hormone-related protein expression in vivo in the rat" *transplantation*, vol 65, 860-863, No. 6, March 27, 1998 (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW) .

(g) Richard, et al. "Humoral Hypercalcemia of Malignancy, Severe Combined Immunodeficient/Beige Mouse Model of Adult T-Cell Lymphoma Independent of Human T-Cell Lymphotropic Virus Type-1 Tax Expression" *Am J Pathol*. 2001 June; 158(6): 2219-2228 (submitted November 27, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at February 23, 2009 in PTO IFW).

(h) Burton et al., "Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma" 1990, *Biochemical and Biophysical Research Communications*, Vol. 167, No. 3, pages 1134-1138 (submitted September 21, 2005 and acknowledged to have been considered by Examiner with

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

signed and dated PTO 1449 Form listing same indexed at January 24, 2007 in PTO IFW).

(i) Ogata et al (EP1197225) (submitted September 21, 2005 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at January 24, 2007 in PTO IFW).

(j) Hoare et al "Specificity and stability of a new PTH1 receptor antagonist, mouse TIP(7-39)" Peptides, 2002, vol 23, No. 5, pp 989-998 (submitted September 21, 2005 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at January 24, 2007 in PTO IFW).

(k) Sato et al (U.S. Patent No. 6,903,194) (submitted January 10, 2008 and acknowledged to have been considered by Examiner with signed and dated PTO 1449 Form listing same indexed at March 6, 2008 in PTO IFW).

(l) Declaration of MASSFELDER executed February 14, 2008 and present in PTO IFW indexed at February 14, 2008 (filed prior to non-final Office Action of March 6, 2008).

(m) In re Smith 173 USPQ 679 (CCPA 1972) (cited by Examiner on page 4 of the Office Action dated February 23, 2009 and page 4 of the Office Action dated August 1, 2008).

(n) In re Ahlbrecht (168 USPQ 293 (CCPA 1971) (discussed in applicants Remarks of the Amendment filed November 27, 2008 and acknowledged to have been considered by Examiner in Office Action dated February 23, 2009).

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

(o) In re Risse (154 USPQ 1 (CCPA 1967) (discussed in applicants Remarks of the Amendment filed November 27, 2008 and acknowledged to have been considered by Examiner in Office Action dated February 23, 2009).

(p) Capon v. Eshhar, 76 USPQ.2d 1078, 1084 (Fed. Cir. 2005) (discussed in applicants Remarks of the Response filed April 13, 2009 and acknowledged to have been considered by Examiner in Advisory Action dated May 1, 2009).

(q) In re Kenneth Alonso (2008-1079 Fed. Cir. October 30, 2008) (discussed in applicants Remarks of the Response filed April 13, 2009 and acknowledged to have been considered by Examiner in Advisory Action dated May 1, 2009).

(r) Enzo Biochem v. Gen-Probe, Inc., 63 USPQ2d 1609, 1613 (Fed. Cir. 2002) (discussed in applicants Remarks of the Response filed April 13, 2009 and acknowledged to have been considered by Examiner in Advisory Action dated May 1, 2009).

(s) Noelle v. Lederman, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004) (discussed in applicants Remarks of the Response filed April 13, 2009 and acknowledged to have been considered by Examiner in Advisory Action dated May 1, 2009).

(t) Calbiochem product sheet (discussed in applicants Remarks of the Response filed April 13, 2009 and acknowledged to have been considered by Examiner in Advisory Action dated May 1, 2009).

(u) Rule 132 DECLARATION of Dr. OULAD ABDELGHANI executed November 27, 2008 (filed November 27, 2008 and acknowledged to have been considered by the Examiner on page 3 of the Office Action dated February 23, 2009).

MASSFELDER et al.
Serial No. 10/520,085
Atty. Ref.: 3665-133
Appeal Brief
August 28, 2009

(10) RELATED PROCEEDINGS APPENDIX

Attached:

NONE

Immunohistochemical Localization of Parathyroid Hormone-related Protein in Canine Mammary Tumors

H. OKADA, Y. NISHIUMA, T. YOSHINO, A. GRÖNE,
C. C. CAPEEN, AND T. J. ROSOL

Abstract. Parathyroid hormone-related protein (PTHrP) was localized immunohistochemically in 58 canine mammary tumors (31 malignant, 27 benign) and adjacent normal or hyperplastic mammary tissue. PTHrP immunoreactivity was significantly enhanced by pretreatment with microwave heating in normal and neoplastic tissues. Epithelial cells of hyperplastic and neoplastic mammary tissues, myoepithelial cells, and metaplastic osteoblasts in mammary tumors stained moderately to strongly positive for PTHrP. No significant difference between staining intensity for PTHrP and histologic pattern of mammary tumors was found. The presence of PTHrP in normal and neoplastic canine mammary tissues supports a pathophysiological role for PTHrP as a paracrine or autocrine hormone in the mammary gland.

Key words: Antigen retrieval; dogs; immunohistochemistry; mammary tumor; parathyroid hormone-related protein.

Humoral hypercalcemia of malignancy (HHM) is an important paraneoplastic syndrome associated with certain malignant tumors, including squamous, renal, and mammary carcinomas, in humans and animals.¹⁻¹⁰ Parathyroid hormone-related protein (PTHrP) is a recently identified polypeptide hormone derived from human and animal tumors that plays a central role in the pathogenesis of HHM.^{4,9} PTHrP is composed of 139, 141, or 173 amino acids.⁹ The N-terminus (1-36) and midregion (36-111) of PTHrP are well conserved among species, suggesting there is immunologic cross-reactivity between species.⁹ Eight of the first 13 N-terminal amino acids of PTHrP are identical to those of parathyroid hormone (PTH). The first 34 amino acids of PTHrP contain PTH-like biological activity and act in kidney and bone to increase osteoclastic bone resorption, renal calcium resorption, and urinary phosphate excretion in patients with HHM.⁹

Breast cancer in humans frequently metastasizes to bone and may be associated with HHM.^{7,9,10} In some patients with breast cancer, increased serum concentrations of PTHrP and hypercalcemia may occur without evidence of bone metastasis. Immunoreactive PTHrP and PTHrP messenger RNA have been detected in 60% of primary breast tumors of humans.^{7,10} Production of PTHrP by mammary cancer in humans is likely important in the pathogenesis of HHM that occurs in certain patients and may play a role in the pathogenesis of metastasis to bone. In contrast, mammary cancer in animals has a low incidence of bone metastasis and hypercalcemia is rare. In dogs, a high percentage of mammary tumors are benign, and many tumors contain mixed cell types, including epithelial cells, myoepithelial cells, and metaplastic bone or cartilage.

PTHrP is present in many normal tissues, including the lactating mammary gland, where it functions as a paracrine or autocrine hormone. High concentrations of PTHrP are present in human, bovine, porcine, murine, rat, and opossum milk.^{1,9} The concentration of PTHrP in bovine milk has been shown to correlate positively with total milk calcium concentration.⁹ PTHrP has been identified immunohistochemically in human lactating mammary tissue (epithelial cells of

the alveoli and ducts).^{4,7} PTHrP immunoreactivity in dogs also has been reported in ductular epithelial cells and myoepithelial cells of nonlactating mammary tissue.⁵ PTHrP may play an important physiological role in lactation and neonatal calcium balance or development.⁹

Immunostaining for PTHrP with trypsin pretreatment unmasked reactive epitopes in paraffin-embedded sections and enhanced the immunoreactivity of chicken-derived polyclonal antibody to PTHrP (1-36) in normal and neoplastic canine tissues.⁵ However, trypsinization uniformly decreased the immunostaining reactivity when a commercially available rabbit-derived anti-PTHrP (34-53) antibody was used in canine tissues.⁵ The purpose of the present study was to investigate the localization of immunoreactive PTHrP in mammary tumors and adjacent nonneoplastic mammary tissue of dogs and to evaluate the effect of microwave pretreatment on PTHrP immunoreactivity in formalin-fixed, paraffin-embedded sections.

Fifty-eight surgical specimens from canine mammary tumors were collected from 1989 to 1993 (Table 1). Mammary tissues including tumor were fixed in 10% buffered formalin (pH 7.4) and embedded routinely in paraffin. Serial sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides and stained with hematoxylin and eosin or stained immunohistochemically using an avidin-biotin-peroxidase complex method.⁵ Histopathologic diagnoses were made based on the World Health Organization classification. For immunohistochemistry, dewaxed sections were treated with and without trypsin or microwave heating. Tissues were heated in a 10 mM citric acid solution (pH 6.0) in a household microwave at 600 W twice for 5 minutes, with a 1 minute interval.⁵ For trypsinization, sections were digested in 0.1% trypsin (Difco, Detroit, MI) in Tris-HCl buffer solution (pH 7.6) containing 0.1% calcium chloride for 30 minutes at 37°C. Primary antibodies consisted of polyclonal chicken anti-human PTHrP (1-36) IgG (1:30) and polyclonal rabbit anti-human PTHrP (34-53) IgG (1:30, Oncogen Science, Manhasset, NY). Characterization and specificity of chicken anti-human PTHrP (1-36) IgG have been described previously.⁵ As a positive control, a serially trans-

Table 1. Histopathologic classification (according to WHO) of 58 canine mammary tumors.

Tumor Type	No. Cases
Malignant tumors	
Tubular simple adenocarcinoma	1
Tubular complex adenocarcinoma	18
Papillary complex adenocarcinoma	1
Simple solid carcinoma	3
Complex spindle cell carcinoma	1
Osteosarcoma	2
Malignant mixed tumor	5
Benign tumors/dysplasia	
Simple adenoma	1
Complex adenoma	8
Ductal papilloma	2
Papillary cyst	1
Duct ectasia	4
Ductal hyperplasia	2
Benign mixed tumor	9

plantable tumor (CAC-8) in nude mice that produced PTHrP and was derived from a canine apocrine adenocarcinoma of the anal sac was stained immunohistochemically for PTHrP. Negative controls were incubated with nonimmune rabbit and chicken sera (1:30 dilution), phosphate-buffered saline (PBS), or anti-human PTHrP IgG mixed with an excess of PTHrP (1-36) instead of the primary antibody.

Without any pretreatment, immunoreactivity of PTHrP (1-36) and PTHrP (34-53) was weak and moderately positive in the tissues. After trypsinization, immunoreactivity of PTHrP (1-36) increased slightly, whereas immunostaining for PTHrP (34-53) decreased in the CAC-8 adenocarcinoma and canine mammary tissues. Pretreatment with microwave heating enhanced immunoreactivity for both PTHrP antibodies in all tissues as compared with immunostaining with or without trypsin (Figs. 1, 2). Moreover, immunostaining patterns of PTHrP (1-36) and PTHrP (34-53) were identical in normal and neoplastic tissues when sections were stained following microwave irradiation. Therefore, the results of immunohistochemical staining for PTHrP are described with microwave pretreatment.

In normal mammary tissue adjacent to the neoplasms, the alveolar and ductal epithelial cells stained moderately to strongly positive for PTHrP and were surrounded by a layer of moderately positive myoepithelial cells (Fig. 1). Positive immunoreactivity for PTHrP also was present in epidermal keratinocytes, epithelial and myoepithelial cells of apocrine sweat glands, sebaceous glands, arteriolar smooth muscle, and skeletal muscle.

In all benign and malignant mammary tumors and dysplastic lesions, mammary epithelial cells and myoepithelial cells were moderately positive for PTHrP (1-36) and PTHrP (34-53). In complex carcinomas and benign or malignant mixed tumors, neoplastic myoepithelial cells stained intensely positive for PTHrP (Figs. 3, 4). PTHrP immunoreactivity was intense in metaplastic chondrocytes of benign and malignant mixed tumors (Fig. 4). In an osteosarcoma arising in

a mixed mammary tumor, neoplastic osteoblasts stained moderately positive for PTHrP. Immunoreactive PTHrP was not present in the matrix of metaplastic cartilage or bone in benign or malignant mixed tumors or in the bone matrix of an osteosarcoma. All control sections stained with nonimmune rabbit serum, chicken IgG, or PBS were negative. Immunostaining intensity for PTHrP using anti-human PTHrP (1-36) IgG mixed with an excess of PTHrP (1-36) was markedly diminished.

This investigation demonstrated that both N-terminal and midregion PTHrP were localized immunohistochemically in normal, hyperplastic, dysplastic, and neoplastic canine mammary tissues after microwave pretreatment. Immunoreactive PTHrP has been identified in many normal and neoplastic human and animal tissues, including the mammary glands.³⁻¹⁰ Immunohistochemical distribution of PTHrP in normal tissues of the dog is identical to that in human tissues.³ The wide distribution of PTHrP in various normal and neoplastic tissues is associated with very low serum PTHrP concentrations, which suggests that PTHrP functions in an autocrine or paracrine manner in most tissues. However, neoplasms that secrete high levels of biologically active PTHrP may induce the syndrome of HHM. In a previous study, immunoreactive PTHrP was not detected in mammary alveolar epithelial cells in dogs,¹ but PTHrP immunostaining has been detected in mammary alveolar cells of humans.^{6,7} Pretreatment microwave heating of paraffin-embedded sections enhanced the immunoreactivity for chicken-derived anti-PTHrP (1-36) and for rabbit-derived anti-human PTHrP (34-53) antibodies when compared with trypsin digestion. Microwave heating may have intensified PTHrP immunoreactivity in the present study by unmasking the reactive epitopes between amino acids 1-36 and 34-53 of canine PTHrP. The uniformly positive immunostaining for PTHrP in this study obtained after microwave pretreatment may damage certain epitopes of the PTHrP molecular and/or induce unmasking of antigenic sites.¹

The function of PTHrP in the normal mammary gland is currently unknown. The lactating mammary gland produces PTHrP, which is present in milk at concentrations 10,000-fold greater than that in plasma and increases with duration of lactation.¹ PTHrP may be important in calcium transport across mammary epithelium, in differentiation of the mammary gland, and in calcium homeostasis or intestinal cell differentiation in neonates.⁹ PTHrP also may be significant in relaxation of myoepithelial cells and expansion of mammary alveoli in lactating mammary glands.⁹

PTHrP was originally isolated from human and animal tumors associated with HHM.^{4,9} Mammary carcinomas in humans are occasionally associated with HHM.^{4,8,10} However, the expression of immunoreactive PTHrP and its messenger RNA has been demonstrated in a high percentage of human breast cancer cases.⁷ The functional role of PTHrP in breast cancers is unknown, but PTHrP may play a role in the regulation of cancer metastasis to bone or growth of bone metastases in humans.⁸ Immunoreactive PTHrP has been demonstrated in malignant tumors from both normocalcemic and hypercalcemic dogs,⁵ which suggests that PTHrP expression in tumors has a pathophysiologic role independent of the induction of HHM.³

In summary, immunoreactive PTHrP was detected in nor-

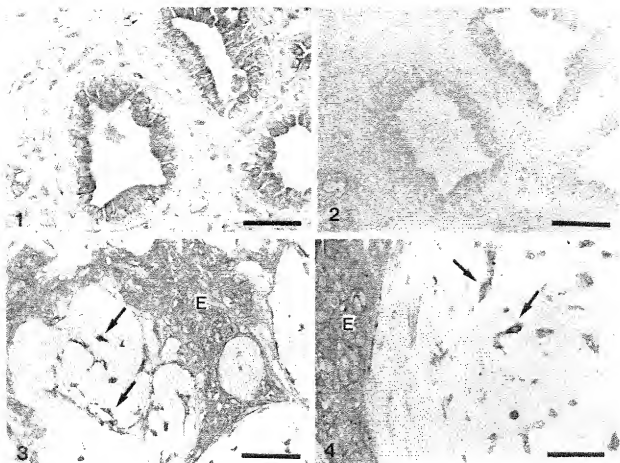


Fig. 1. Mammary gland; dog. Moderately positive staining of acinar epithelium for PTHrP (1–36) treated with microwave heating. Avidin–biotin complex method, methyl green counterstain. Bar = 50 μ m.

Fig. 2. Mammary gland; dog. Extremely weak staining of acinar epithelium for PTHrP (1–36) without microwave pretreatment. Avidin–biotin complex method, methyl green counterstain. Bar = 50 μ m.

Fig. 3. Mammary complex tubular adenocarcinoma; dog. Moderately positive staining of neoplastic epithelial cells (E) and strong immunostaining of myoepithelial cells (arrows) for PTHrP (1–36) with microwave pretreatment. The intercellular matrix is negative for PTHrP. Avidin–biotin complex method, methyl green counterstain. Bar = 50 μ m.

Fig. 4. Benign mixed mammary tumor; dog. Moderately positive staining of neoplastic epithelial cells (E), strong immunostaining of metaplastic chondrocytes (arrows), and negative staining of cartilage matrix for PTHrP (1–36) treated with microwave heating. Avidin–biotin complex method, methyl green counterstain. Bar = 50 μ m.

mal, hyperplastic, dysplastic, and neoplastic mammary canine tissues. However, there was no difference in intensity of immunostaining, distribution of PTHrP, and histologic features between benign and malignant tissues.

Acknowledgements

This work was partly supported by a Grant-in-Aid for International Scientific Research (University to University Co-operative Research, no. 08045059) and a Grant-in-Aid for Scientific Research (C) (no. 08660369) from The Ministry of Education, Science, Sports and Culture of Japan and by State of Ohio Canine Research Fund (grant no. 611424).

References

- 1 Budayr AA, Hallpran BP, King JC, Diep D, Nissenson RA, Strewler GJ: High levels of a parathyroid hormone-like protein in milk. *Proc Natl Acad Sci USA* **86**:7183–7185, 1989
- 2 Cattorretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J: Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB1 and MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* **168**:357–363, 1992
- 3 Danks JA, Ebeling PR, Hayman J, Chou ST, Moseley JM, Dunlop J, Kemp BE, Martin TJ: Parathyroid hormone-related protein: immunohistochemical localization in cancers and in normal skin. *J Bone Miner Res* **4**:273–278, 1989
- 4 Edwards RC, Ratcliffe WA, Walls J, Morrison JM, Ratcliffe JG, Holder R, Bundred NJ: Parathyroid hormone-

- related protein (PTHrP) in breast cancer and benign breast tissue. *Eur J Cancer* **31A**:334-339, 1995
- 5 Gröne A, Werkmeister JR, Steinmeyer CL, Capen CC, Rosol TJ: Parathyroid hormone-related protein in normal and neoplastic canine tissue: immunohistochemical localization and biochemical extraction. *Vet Pathol* **31**: 308-315, 1994
 - 6 Kramer S, Reynolds FH Jr, Castillo M, Valenzuela DM, Thorikay M, Sorvillo JM: Immunoidentification and distribution of parathyroid hormone-like protein polypeptides in normal and malignant tissues. *Endocrinology* **128**:1927-1937, 1991
 - 7 Liapis H, Crouch EC, Grosso LE, Kitazawa S, Wick MR: Expression of parathyroidlike protein in normal, proliferative, and neoplastic human breast tissues. *Am J Pathol* **143**:1169-1178, 1993
 - 8 Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ: Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res* **51**:3059-3061, 1991
 - 9 Rosol TJ, Capen CC: Biology of disease. Mechanisms of cancer-induced hypercalcemia. *Lab Invest* **67**:680-702, 1992
 - 10 Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, Bennett RC, Martin TJ: Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res* **50**:7710-7716, 1990
- Request reprints from Dr. H. Okada, Department of Veterinary Pathology, Rakuno Gakuen University, Ebetsu, Hokkaido 069 (Japan).

Vet Pathol **34**:359-363 (1997)

Rhabdoid Tumor in the Brain of a Dog

K. E. STEELE, F. Y. SCHULMAN, H. MENA, AND E. O. STIMPLE

Abstract. Rhabdoid tumor is a neoplasm of uncertain cellular origin recognized in humans. These tumors most commonly arise in the kidneys of children, but they can also affect many extrarenal sites, including the central nervous system. Similar neoplasms have not been reported in nonprimate species. A malignant brain tumor in a young dog was characterized by large cells with globular intracytoplasmic inclusions composed of intermediate filaments. By immunohistochemistry, neoplastic cells were uniformly reactive for vimentin and demonstrated scattered reactivity for glial fibrillary acidic protein and neuron-specific enolase. The intermediate filaments also reacted with vimentin antibodies by immunogold electron microscopy. The findings in this case are remarkably similar to the histologic, ultrastructural, and immunocytochemical features of rhabdoid tumors in humans.

Key words: Brain neoplasms; dogs; immunohistochemistry; rhabdoid tumor; ultrastructure.

Rhabdoid tumor (RT) is an aggressive, malignant neoplasm that is recognized in humans and that most commonly arises in the kidneys of children.^{1,12} RTs have also been reported as primary neoplasms in extrarenal locations, including the central nervous system (CNS).⁹ We are aware of only one report of an RT in a nonhuman species; this RT affected the stomach of an aged orangutan.⁵

Although the cellular origin of this neoplasm is controversial, the diagnosis of rhabdoid tumor is based on the characteristic histologic, ultrastructural, and immunohistochemical features.^{4,6,7,8,12} Histologically, RTs are typically composed of large polygonal to globoid eosinophilic cells with globular intracytoplasmic inclusions. Nuclei are vesicular, often with margined chromatin, and contain large distinct nucleoli. Ultrastructurally, the cytoplasmic inclusions are composed of discrete aggregates of intermediate filaments, usually arranged in whorls. By immunohistochemistry, the intermediate filaments react consistently with antisera to vimentin. Less frequently, cells stain using antisera to cyto-

keratins, epithelial membrane antigen (EMA), glial fibrillary acidic protein (GFAP), or neuron-specific enolase (NSE). We report here on a neoplasm with features of the human rhabdoid tumor in a dog.

A 1.5-year-old female Border Collie presented with a 2-week history of seizures, drooling, and weakness in the hind limbs. Complete blood count and blood chemistry evaluations were within normal limits. Vaccinations for typical canine diseases, including rabies, had been performed in the previous year. Previously, the dog had not exhibited any evidence of health problems. Antiseizure therapy with phenobarbital (10 mg/kg twice daily) was initiated, but the clinical signs gradually progressed to include incoordination and circling. Two months later, the dog exhibited circling to the right, left hind limb hemiplegia, and, based on a lack of menace response, appeared blind in the left eye. The dog was euthanized, and the brain was removed for examination. Other organs were not examined.

The right pyriform lobe exhibited a soft gray-tan mass



Parathyroid hormone-related peptide (PTHrP) induces parietal endoderm formation exclusively via the Type I PTH/PTHrP receptor

Mark H.G. Verheijen^a, Marcel Karperien^{a,b}, Ung-il Chung^c, Merel van Wijnen^a,
Heleen Heystek^a, Jeanine A.A. Hendriks^a, Jacqueline M. Veltmaat^a, Beate Lanske^c,
En Li^d, Clemens W.G.M. Löwik^b, Siegfried W. de Laat^a,
Henry M. Kronenberg^c, Libert H.K. Defize^{a,*}

^aHabrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

^bDepartment of Endocrinology, University Hospital Leiden, Leiden, The Netherlands

^cEndocrine Unit, Massachusetts General Hospital, Boston, MA, USA

^dCardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA

Received 20 July 1998; accepted 16 December 1998

Abstract

A number of studies suggest a role for PTHrP and the classical PTH/PTHrP receptor (type I) in one of the first differentiation processes in mouse embryogenesis, i.e. the formation of parietal endoderm (PE). We previously reported that although in type I receptor (–/–) embryos PE formation seemed normal, the embryos were smaller from at least day 9.5 p.c. and 60% had died before day 12.5 p.c. Here we show that the observed growth defect commences even earlier, at day 8.5 p.c. Using two novel antibodies, we show that the expression of the type I receptor protein at this stage is confined to extraembryonic endoderm only. In addition, we show that large amounts of PTHrP protein are present in the adjacent trophoblast giant cells, suggesting a paracrine interaction of PTHrP and the type I PTH/PTHrP receptor in PE formation. The involvement in PE differentiation of other recently described receptors for PTHrP would explain a possible redundancy for the type I receptor in PE formation. However, deletion of the type I PTH/PTHrP receptor in ES cells by homologous recombination completely prevents PTHrP-induced PE differentiation. Based upon these observations, we propose that PTHrP and the type I PTH/PTHrP receptor, although not required for the initial formation of PE, are required for its proper differentiation and/or functioning. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Embryonal stem cells; Homologous recombination; Immunohistochemistry; Parathyroid hormone/parathyroid hormone-related peptide receptor; Parathyroid hormone-related peptide; Parietal endoderm

1. Introduction

PTHrP was initially identified in tumors associated with humoral hypercalcemia of malignancy, and was since then shown to be important in a wide range of growth and differentiation processes (reviewed in Mallette, 1991; Orloff et al., 1994). The N-terminus of PTHrP, PTHrP(1–34), contains the PTH-like portion of this molecule, and is comparable with PTH(1–34) in binding and activation of the

classical PTH/PTHrP receptor (type I) (Abou-Samra et al., 1992). While PTH and the type I receptor are known to serve an important role in regulation of calcium homeostasis in the adult organism (reviewed in Mallette, 1991), we and others have suggested a role for PTHrP and the type I receptor in the formation of parietal endoderm (PE) in the early mouse embryo (Chan et al., 1990; van de Stolpe et al., 1993; Karperien et al., 1994, 1996; Behrendtsen et al., 1995). However, mice in which the type I receptor gene was ablated did not show obvious abnormalities in PE formation (Lanske et al., 1996). Histological analysis of the PE of these mice suggested that this cell layer developed normally, although the mice were smaller than normal from at least

* Corresponding author. Tel.: +31-302510211; fax: +31-302516464;
e-mail: bus@nio.b.knaw.nl

day 9.5 p.c., while, depending on the genetic background, 60% had died at day 12.5 p.c.

It was thought for a long time that the type I PTH/PTHrP receptor was involved in almost all of the actions of the N-terminal PTH and PTHrP fragments. However, with the recent discovery of additional receptors for N-terminal, midregion and C-terminal fragments of PTH and/or PTHrP, this concept is no longer tenable (Fenton et al., 1991; Fukayama et al., 1995; Orloff et al., 1995; Usdin et al., 1995; Kovacs et al., 1996; Yamamoto et al., 1997). The presence of these other receptors for PTH and/or PTHrP might explain the residual PE formation in type I receptor ($-/-$) embryos.

Herc, we present data which indicate that PTHrP exerts its actions in PE formation exclusively through the type I receptor, and furthermore suggest that PTHrP and the type I receptor are required for proper differentiation and/or functioning of PE.

2. Results

2.1. Type I PTH/PTHrP receptor ($-/-$) embryos are growth retarded at day 8.5 p.c.

We previously reported that type I receptor ($-/-$) embryos are smaller from at least day 9.5 p.c. (Lanske et al., 1996). To examine the growth retardation during this stage of development in more detail, we directly measured the length of isolated embryos, and also included 8.5 p.c. embryos in the analysis. For 9.5 p.c. embryos the crown-rump length was measured (Brown, 1990). Since for day 8.5 p.c. embryos, which have not yet turned this analysis cannot be performed, the length of such embryos was measured differently, i.e. as the distance between a line touching the tips of the U-shaped embryo and a line touching the bottom of the U. Importantly, there was no difference in the curvature between wt, $+/-$ or $-/-$ embryos. Only embryos which had not turned yet were analyzed. No differences were observed between the length of wildtype and heterozygous embryos, while 90% of the type I receptor ($-/-$) embryos were shorter than wildtype/heterozygous embryos at both day 8.5 as well as day 9.5 p.c. (Table 1). The shorter length

of ($-/-$) embryos was already significant at day 8.5 p.c., and became even more pronounced at day 9.5 p.c. Type I receptor ($-/-$) embryos showed no significant differences in the number of somites compared to wildtype/heterozygous embryos (data not shown). Thus type I receptor ($-/-$) embryos are already growth retarded at day 8.5 p.c., suggesting defective formation of the extraembryonic endoderm, the only site of receptor mRNA expression at this stage (Karperien et al., 1994).

2.2. PTHrP-induced PE differentiation of ES cells is dependent on the type I PTH/PTHrP receptor

To investigate the possible involvement of other receptors for PTH and/or PTHrP than the type I PTH/PTHrP receptor in PE differentiation, we determined the effect of different PTH and PTHrP fragments on PE differentiation of wild type ES cells and of ES cells with a homozygous deletion of the type I PTH/PTHrP receptor gene established from type I PTH/PTHrP receptor knockout mice (see Section 4). In this study, we performed experiments with the wildtype ES cell line 9(+/-) and the knock out ES cell lines 1($-/-$) and 5($-/-$). Since no experimental differences were observed between ES 1 ($-/-$) and 5 ($-/-$), only results with ES 5 ($-/-$) are shown. Differentiation to PE was determined using the PE marker thrombomodulin (Imada et al., 1987; Weiler-Guettler et al., 1996) and by the changes in morphology (Strickland and Mahdavi, 1978; Strickland et al., 1980; van de Stolpe et al., 1993). No expression of thrombomodulin could be detected in undifferentiated ES cells. However, addition of RA for 6 d induced low expression of thrombomodulin in ES(9+/-) cells (Fig. 1A). Thrombomodulin was expressed at sites of high confluency by a few cells with a PE-like morphology (small and rounded cells with long filopodia) and not by the majority of the cells with a PrE-like morphology (flattened and epithelial-like cells) (see Fig. 4 and Fig. 5B). Treatment with the N-terminal fragments, PTH(1-34) and PTHrP(1-34), or the full length versions of these molecules, PTH(1-84) and PTHrP(1-141), induced a PE-like morphology and a strong increase in thrombomodulin expression (Figs. 1A and 5B). Quantification by densitometric scanning of the autoradiogram in Fig. 1A revealed these increases to be 4-

Table 1
Relative length of type I PTH/PTHrP receptor ($-/-$) embryos

Days of gestation	Total no. of embryos	($-/-$) embryos		
		n	Smaller ^a	Relative length (%) ^b
8.5	43	10	9	83.3 ± 4.1 ^a
9.5	49	10	9	77.5 ± 4.6 ^{a*}

^aLength of embryos was determined using a micrometer eyepiece, and number of ($-/-$) embryos with length smaller than the average length of wildtype and heterozygous embryos is given.

^bLength of ($-/-$) embryos as percentage of the average length of wildtype and heterozygous embryos. Data are the mean ± SEM.

* $P < 0.01$, ** $P < 0.001$. Analysis of difference between ($-/-$) embryos and wildtype plus heterozygous embryos was performed using Bonferroni t -test with the mean square error of two-way ANOVA as error variance.

to 5-fold compared to treatment with RA alone, while dbcAMP elevated thrombomodulin levels approximately 20-fold. Importantly, treatment with PTHrP(67–86) (not shown), PTHrP(67–94) or PTHrP(107–139) did not affect thrombomodulin expression nor did it induce a PE-like morphology. Thus, PTHrP induces PE differentiation via its N-terminal part and receptors for the midregion or C-terminal PTHrP fragments are not involved in PTHrP-induced PE differentiation.

As observed for the wildtype ES cells, treatment of ES 5(–/–) cells with RA for 6 days induced low amounts of thrombomodulin (Fig. 1B), which was expressed at sites of high confluency by a few cells with a PE-like morphology. Subsequent treatment with the different PTH(rP) fragments during the last 3 days did not elevate thrombomodulin expression (Fig. 1B) nor did it increase the amount of cells with a PE-like morphology. Importantly, dbcAMP was perfectly able to induce thrombomodulin expression (Fig. 1B) and a PE-like morphology (Fig. 5B), showing that the ES5(–/–) cells were not impaired in their capacity to form PE. Thus, the type I receptor is necessary for the PTHrP-induced PE differentiation. Furthermore, the observation that treatment with RA is sufficient to induce some PE differentiation in ES 5(–/–) cells suggests that this 'spontaneous' differentiation to PE is not caused by an autocrine mechanism involving PTHrP and the type I PTH/PTHrP receptor, as was previously suggested (van de Stolpe et al., 1993). We therefore conclude that the type I PTH/PTHrP receptor is essential for the PTHrP-induced PE differentiation of ES cells, and that other mechanisms can contribute to PE differentiation as well.

2.3. Characterization of anti-type I PTH/PTHrP receptor antibodies

Two novel antibodies A27 and P31, directed against, respectively, the extracellular N-terminus and the intracellular C-terminus of the receptor (see Section 4) were tested by Western blotting using cell extracts of COS or 293 cells transiently transfected with mouse type I receptor expression vector (cMR13). Both P31 as well as A27 recognized a band with the expected molecular weight of approximately 85 kDa in 293 cells (Fig. 2A) and COS cells (Fig. 2B) transfected with cMR13 but not in mock transfected cells (Karpf et al., 1987; Shigeno et al., 1988). A band of approximately 160–180 kDa could be detected also and might present a dimer of the receptor. However, other bands with a molecular weight of approximately 60 and 70 kDa were detected in COS cells as well. Since the type I receptor contains four putative N-linked glycosylation sites, the bands of 60 and 70 kDa could represent less glycosylated receptor protein. Inhibition of N-linked glycosylation with tunicamycin resulted in strong expression of the 60 kDa band, which thus likely represents the putative backbone of the type I receptor, and a band of approximately 120–140 kDa which could represent a dimer of unglycosylated

type I receptors (Fig. 2B). Dimerization or microaggregation has previously been demonstrated for the β_2 -adrenergic receptor and the gonadotropin releasing hormone receptor, and shown to be stabilized by agonist treatment (Hebert et al., 1996; Janovick and Conn, 1996). The biological relevance of the putative type I receptor dimers and their existence under more physiological circumstances remains to be determined.

We could not detect any expression of type I PTH/PTHrP

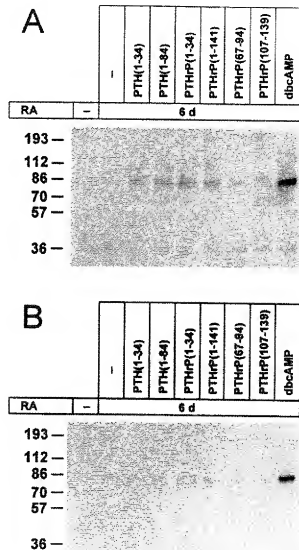


Fig. 1. Effect of PTH and PTHrP analogues on PE differentiation of ES 9(+/+) and ES 5(–/–) cells. ES 9(+/+) cells (A) or ES 5(–/–) cells (B) were left untreated (leftmost lane), or treated with 1 mM RA for 6 days with or without 100 nM PTH (1-34), recombinant hPTH(1-84), PTHrP(1-34), PTHrP(1-141), PTHrP(67-94), PTHrP(107-139), or 1 mM dbcAMP during the last 3 days, as indicated. Cell lysates were taken up in non-reducing sample buffer and boiled. Proteins were separated by SDS-PAGE on a 10% gel, blotted and detected with monoclonal antibody 273–264 against thrombomodulin. Thrombomodulin is detected as a band of approximately 85 kDa.

154

M.H.G. Verheijen et al. / Mechanisms of Development 81 (1999) 151–161

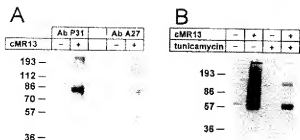


Fig. 2. Detection of the type I PTH/PTHrP receptor in transfected 293 and COS cells. (A) 293 cells were mock transfected (–) or transfected with a mouse type I PTH/PTHrP receptor expression vector (cMR13). Cell lysates were taken up in urea-saturated non-reducing sample buffer, proteins were separated by SDS-PAGE on a 10% gel, blotted and detected with the affinity-purified antibodies, P31 or A27, as indicated. The type I PTH/PTHrP receptor was detected as a band of approximately 85 kDa. (B) COS cells were transfected as described in (A), and treated with tunicamycin (10 mg/ml) or vehicle (DMSO), as indicated. cMR13 is detected with the antibody P31. In mock transfected cells an specific band of approximately 61 kDa is observed.

receptor protein in undifferentiated F9 EC cells, while low, but detectable, expression was found during RA-induced differentiation towards PrE (Fig. 3A). Subsequent differentiation to PE, by addition of PTHrP or dbcAMP, was accompanied by a strong upregulation of receptor expression (Fig. 3A). The molecular weight of the receptor in PrE and PE-like cells was 85 kDa, suggesting that it was normally glycosylated during this differentiation process. Differentiation to PE was confirmed by Western blotting on thrombomodulin. As shown in Fig. 3B, thrombomodulin was not expressed, or at very low levels, in undifferentiated or RA-induced PrE-like cells, while subsequent differentiation to PE by treatment with PTHrP or dbcAMP strongly induced thrombomodulin expression. The identity of the cells expressing the type I receptor was determined by immunofluorescence. As expected from the Western blotting experiments, we could not find any expression in undifferentiated cells (Fig. 4), while RA-induced differentiation to a PrE-like phenotype resulted in expression of the receptor at low levels. Treatment of PrE-like cells with PTHrP or dbcAMP strongly induced expression of the type I receptor in cells differentiated to a PE-like phenotype.

Specificity of the antibodies was confirmed using Western blotting and immunofluorescence on ES 9(+/+) and ES 1(–/–) cells. Fig. 5A shows a Western on the type I PTH/PTHrP receptor in differentiating ES cells. No type I PTH/PTHrP receptor expression was detected in undifferentiated ES cells (not shown). Treatment with RA induced a strong increase in receptor protein expression of the ES 9(+/+) cells, while subsequent treatment with PTHrP(1-34) or dbcAMP induced a further increase in expression. No expression was found in ES 5(–/–) cells (Fig. 5A). The sharp band of approximately 80 kDa, which in ES 9(+/+) cells runs close to the receptor band of 85 kDa, can be detected in ES 5(–/–) cells as well, suggesting that it is recognized non-specifically. The phenotype of the cells

expressing the type I receptor was determined using immunofluorescence. As shown in Fig. 5B, receptor expression was only observed in ES 9(+/+) cells with a PE-like morphology. Again, no receptor staining could be detected in ES 5(–/–) cells, before or after differentiation by RA and dbcAMP, confirming once more the specificity of the antibody.

2.4. The type I PTH/PTHrP receptor protein is expressed in parietal and visceral endoderm of early implantation embryos

To investigate whether the type I receptor protein is indeed expressed at sites involved in PE differentiation, we performed immunohistochemistry with both A27 and P31 antibodies. The earliest stages in which we observed expression were early postimplantation embryos of approximately day 5.5 p.c. The embryos were left in utero and embedded as such in paraffin, or were frozen in tissue freezing medium, sectioned and stained for the type I PTH/PTHrP receptor with antibodies P31 and A27, as described in Section 4. As shown in Fig. 6A, the receptor was detected at high levels in tissue of both embryonal and maternal origin. This staining was specific since both antibodies showed identical staining patterns, while no staining was observed when the first antibody was omitted (not shown). Maternal type I receptor was expressed in the outer zone of the decidua and in the inner circular myometrial smooth muscle layers. This expression pattern is consistent with a proposed role for PTHrP in relaxation of uterine smooth muscles and inhibition of uterus contractility before partus (Thiede et al., 1991; Daifotis et al., 1992; Paspaliaris et al., 1992; Williams et al., 1994). Embryonic type I PTH/PTHrP receptor was detected only in the extraembryonic cell layers, being PE and VE (Fig. 6B). A comparable expres-

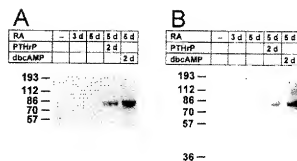


Fig. 3. Expression of the type I PTH/PTHrP receptor protein (A) and thrombomodulin (B) during endoderm differentiation of F9 EC cells. F9 cells were left untreated, treated with 1 mM RA for 3 days to induce PrE differentiation, or treated with 1 μ M RA for 5 days with or without 100 nM PTHrP(1-34) or 1 mM dbcAMP during the last 2 days to induce PE differentiation. (A) Samples were treated as described in the legend of Fig. 2. The type I PTH/PTHrP receptor was detected with antibody P31. Similar results were obtained with the antibody A27 (not shown). (B) Samples were treated as described in legend of Fig. 1. Immunoblots with antibody 273-34A against thrombomodulin are shown.

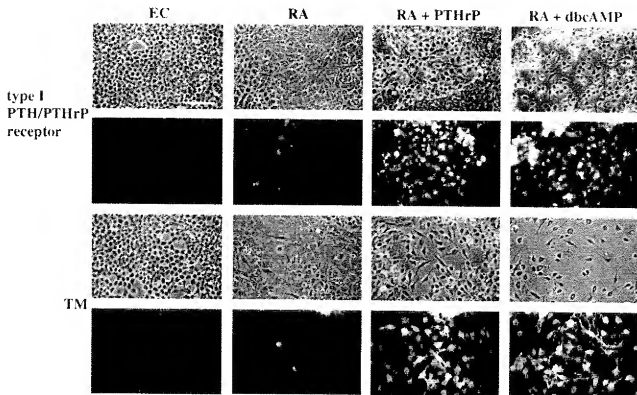


Fig. 4. Immunofluorescent staining of the type I PTH/PTHrP receptor and thrombomodulin during endoderm differentiation of F9 EC cells. F9 cells were left untreated (EC), or treated with 1 mM RA for 5 days with or without 100 nM PTHrP(1-34) or 1 mM dbcAMP during the last 2 days as indicated. Shown are the immunofluorescent staining of the type I PTH/PTHrP receptor, with antibody P31, or of thrombomodulin (TM), as described in Section 4. Scale bar, 50 μ m.

sion pattern was observed for the type I PTH/PTHrP receptor in the 6.5 day p.c. embryo (Fig. 6D), while only background staining was detected in the control (staining procedure when first antibody was omitted, Fig. 6C). Interestingly, high levels of receptor expression were found in the marginal zone, a region where PE and VE are in close contact, and from which PE cells are recruited (Hogan and Newman, 1984). The visceral endoderm cells near the marginal zone have been demonstrated to possess the capacity to differentiate to PE cells until day 7.5 p.c. (Hogan and Tilly, 1981; Cockroft and Gardner, 1987). It was at this stage that we observed a decrease in expression of the type I PTH/PTHrP receptor in VE, compared to earlier stages, while expression in PE was still high (Fig. 6F). No expression could be detected at other sites, although some staining was detected in embryonic tissue between the ectoderm and mesoderm, which was however non-specific since a similar staining was observed in the control (Fig. 6E). Type I receptor (–/–) embryos of day 8.5 and 9.5 p.c. are smaller than normal (see above). Importantly, while the only site of expression of the type I PTH/PTHrP receptor protein until 7.5 p.c. was in PE and VE, the only site of expression at day 9.5 p.c. was in PE, which can be recognized as a single cell layer between the trophoblasts giant cells and the visceral yolk sac (Fig. 6G). No staining was detected in the

marginal zone, the VE or in the embryo proper (not shown). This suggests that the phenotype of type I receptor (–/–) embryos, at least the smaller size observed at day 8.5 and 9.5 p.c., is a consequence of a lack of the type I receptor in (developing) PE.

To measure PTHrP protein expression in the 9.5 p.c. embryo we used affinity-purified rabbit anti-PTHrP (34-54) IgG. PTHrP protein was strongly expressed in the trophoblast giant cells (Fig. 6H), while no expression could be detected in embryonic tissues (not shown) nor in VE or PK. Thus, PTHrP and the type I receptor show a complementary expression pattern during early embryonic development.

3. Discussion

ES cells are an established model system for early embryonic events. They are considered to represent undifferentiated inner cell mass cells, and can be differentiated *in vitro* to form derivatives of all three germ layers, including extraembryonic endoderm. PTHrP is an established inducer of PE differentiation *in vitro* (Chan et al., 1990; van de Stolpe et al., 1993). We show here that the PTHrP fragments (67-86), (67-94) and (107-139), for which receptors other than the type I PTH/PTHrP receptor have been described

156

M.H.G. Verheijen et al. / Mechanisms of Development 81 (1999) 151–161

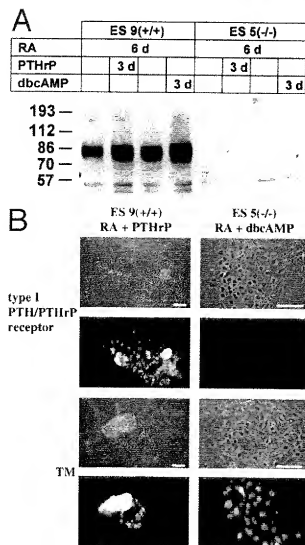


Fig. 5. Staining of the type I PTH/PTHrP receptor and thrombomodulin on differentiated ES (+/+) or ES 5(-/-) cells. ES 9(+/+) and ES 5(-/-) cells were treated with 1 mM RA for 6 days with or without 100 nM PTHrP(1-34) or 1 mM dbcAMP during the last 3 days, as indicated. (A) Samples were treated as described in the legend of Fig. 2. An immunoblot with antibody P31 against the type I PTH/PTHrP receptor is shown. Similar results were obtained with the antibody A27 (not shown). (B) Shown are immunofluorescent staining on the type I PTH/PTHrP receptor, with antibody P31, or on thrombomodulin (TM), as described in Section 4. Scale bar, 50 mm.

(Fenton et al., 1991; Fukuyama et al., 1995; Orloff et al., 1995; Usdin et al., 1995; Kovacs et al., 1996; Yamamoto et al., 1997), do not induce differentiation of ES cells to PE, in contrast to PTHrP fragments containing the N-terminus. Furthermore, the induction of PE by PTHrP(1-34) or PTHrP(1-141) was prevented by homozygous deletion of the type I PTH/PTHrP receptor. Thus, we conclude that PTHrP-induced PE differentiation, at least of ES cells, is mediated exclusively by the type I PTH/PTHrP receptor.

The expression pattern of the type I PTH/PTHrP receptor protein observed *in vivo* was identical to that of its mRNA, as observed by *in situ* hybridization (Karperien et al., 1994). Expression of type I PTH/PTHrP receptor protein was found in PE and VE of day 5.5 and 6.5 p.c. embryos. Interestingly, high amounts of type I PTH/PTHrP receptor protein were detected in the marginal zone, the region where VE and PE are in close contact and from which PE cells are recruited (Hogan and Newman, 1984). Visceral endoderm cells near the marginal zone have been demonstrated to possess the capacity to transdifferentiate into PE cells until day 7.5 p.c. (Hogan and Tilly, 1981; Cockcroft and Gardner, 1987). This correlated with the expression of type I PTH/PTHrP receptor protein found in this study, since expression in VE and the marginal zone decreased at day 7.5 p.c., while it could no longer be detected in these cell layers at day 9.5 p.c. The only site of type I PTH/PTHrP receptor expression at this stage was PE. Type I PTH/PTHrP receptor protein expression was complementary to expression of PTHrP protein in the trophoblast giant cells. The expression patterns of PTHrP and the type I PTH/PTHrP receptor thus strongly confirm the notion that this signaling system is involved in PE formation in a paracrine fashion.

3.1. The role of PTHrP and the type I PTH/PTHrP receptor in PE differentiation *in vivo*

If PTHrP and the type I PTH/PTHrP receptor play a critical role in PE formation, mice with a homozygous deletion for either gene would be expected to die early in gestation because of defects in the parietal yolk sac. However, PTHrP(-/-) mice have developed a seemingly normal PE early in gestation, but die at birth (Karaplis et al., 1994). A more severe phenotype is seen for type I PTH/PTHrP receptor (-/-) mice (Lanske et al., 1996). These mice are smaller than normal at day 8.5 p.c. (this study), while, depending on the genetic background, 60% has died at day 12.5 p.c. Again, no obvious abnormalities are observed in the PE. Although detailed analysis of the PE of these mice is still lacking, the phenotypes suggest that PTHrP and the type I PTH/PTHrP receptor are not essential for formation of PE. Lack of fetal PTHrP in early gestation of PTHrP(-/-) embryos, could likely be compensated for by maternally derived PTHrP. Indeed, high levels of PTHrP mRNA and protein were observed in the maternal tissue surrounding the embryo (Karperien et al., 1996; this study), which might result in transfer of maternal PTHrP to the embryo, a phenomenon described previously for knock outs of other growth factors such as TGF β 1 (Shull et al., 1992). We hypothesized that the lack of a clear phenotype in early gestation of type I PTH/PTHrP receptor (-/-) embryos could be due to compensation by other receptors for PTH or PTHrP. However, our present data clearly show that none of the PTH(rP) fragments could enhance PE differentiation in type I PTH/PTHrP receptor (-/-) ES cells, indicating that at least *in vitro* this was not the case. Our data indicate that

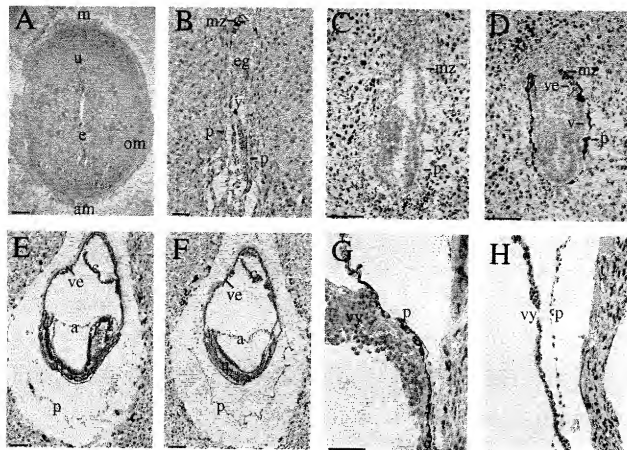


Fig. 6. Expression of PTHrP and the type I PTH/PTHrP receptor protein during early gestation of the mouse. Embryos were left in the uterus, fixed and embedded in paraffin, with the exception of the samples shown in (C,D), which are sagittal sections of embryos quick frozen in tissue freezing medium. The type I PTH/PTHrP receptor protein was detected in A, B, D, F and G with antibody P31, and PTHrP was detected in H, as described in Section 4. Staining is visible in red. (A) Sagittal section of a 5.5 day p.c. embryo. (B) Magnification of the embryo of (A). (C) Sagittal section of a 6.5 day p.c. embryo, control (staining procedure when first antibody was omitted). (D) Sagittal section of a 6.5 day p.c. embryo. (E) Sagittal section of a 6.5 day p.c. embryo, control. (F) Sagittal section of 7.5 day p.c. embryo. (G) Sagittal section of 7.5 day p.c. embryo. (H) Sagittal section of 7.5 p.c. embryo staining of PTHrP. Abbreviations: a, amnion; ac, amniotic cavity; m, mesometrial; e, embryo; eg, egg cylinder; m, mesometrial; m, marginal zone; n, neural tube; om, outer cell layers of decidua and inner circle of mesometrial smooth muscle cells; p, parietal endoderm; t, trophoblast giant cells; u, uterus lumen; v, visceral endoderm; ve, visceral extraembryonic endoderm; vy, visceral yolk sac; y, yolk sac cavity. Scale bars, (A) 100 μ m; (B–D,G,H) 20 μ m; (E,F) 10 μ m.

the type I PTH/PTHrP receptor protein was expressed exclusively in the VE and PE, and not at other sites, until at least day 9.5 p.c. Although it can not be excluded that the type I receptor has a function in extraembryonic VE other than stimulating the formation of PE, the smaller size of the type I PTH/PTHrP receptor ($-/-$) embryos at day 8.5 p.c. strongly suggests that PTHrP and the type I PTH/PTHrP receptor are required for proper PE differentiation and/or functioning. In relation to this, the phenotype of thrombomodulin ($-/-$) embryos is striking, since at day 8.5 p.c. these mice are smaller than normal, while the embryo's failed to survive beyond day 9.5 p.c. Since no obvious pathological abnormalities were observed, and the only site of expression before day 9.5 p.c. was the parietal yolk sac, it was suggested that the failure of thrombomodulin ($-/-$) embryos to survive was consequence of dysfunctional maternal-em-

brionic interaction (Healy et al., 1995). We observed that exogenous PTHrP can strongly increase thrombomodulin expression in RA-treated ES cells. Whether thrombomodulin expression levels are affected in type I PTH/PTHrP receptor ($-/-$) embryos remains to be determined.

Since type I receptor ($-/-$) embryos show formation of PE, the type I receptor is apparently not essential for (initial) PE formation. This is supported by our *in vitro* data using the type I PTH/PTHrP ($-/-$) ES cells. Although these cells do not respond to PTHrP they can still differentiate to PE, indicating that other signals are involved in PE differentiation as well. Recently, Behrendtsen et al. (1995) showed that differentiation and outgrowth of PE from isolated ICM is a cooperative interaction between extracellular matrix, integrins and PTHrP. This suggests that signals induced by extracellular matrix components might compensate for the lack

of PTHrP-induced signals in type I PTH/PTHrP receptor (–/–) embryos.

In conclusion, PTHrP-induced stimulation of PE differentiation is solely mediated by the type I PTH/PTHrP receptor. The expression patterns of the type I PTH/PTHrP receptor protein and the PTHrP protein are strongly consistent with the role for this signaling system as stimulators of PE differentiation *in vivo*. The observation that VE and PE are the only sites of type I PTH/PTHrP receptor expression until at least day 9.5 *p.c.* suggests the phenotype of type I PTH/PTHrP receptor (–/–) embryos at this stage to be explained by improper differentiation and/or functioning of PE. Detailed analysis of PE in these mice will therefore be the subject of further study. Our results are consistent with a model proposed by others, in which PrE cells, of VE cells with PrE-like characteristics, are induced to differentiate into PE and to migrate from the marginal zone over the trophoblast by a cooperation of signals induced by PTHrP and other factors, like extracellular matrix components. We propose that PTHrP, although not required for initial PE differentiation, is essential for proper differentiation and/or function of PE.

4. Experimental procedures

4.1. Materials

dbcAMP was purchased from Aldrich (Zwijndrecht, the Netherlands); ECL from Amersham (Hertogenbosch, The Netherlands); All-trans RA and tunicamycin were from Sigma rat PTH(1-34) was purchased from Peninsula Laboratories Europe (St. Helens, UK); PTHrP(1-34) from Bachem (Bubendorf, Switzerland); PTHrP(67-86), PTHrP(67-94) hPTHrP(107-139) and hPTHrP(1-141) were a gift from Jane Moseley (University of Melbourne, Australia). Recombinant hPTH(1-84) was obtained as described previously (Inomata et al., 1995), using a bacterial expression vector for full length hPTH, kindly provided by Dr. H. Jüppner (Massachusetts General Hospital and Harvard Medical School, Boston, MA). Affinity-purified polyclonal rabbit anti-human PTHrP(34-53) IgG were purchased from Oncogene Science (Mincola, NY). Monoclonal antibodies against thrombomodulin were a gift from Dr. S.J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN).

4.2. Embryos

Embryos homozygous for the PTH/PTHrP receptor gene were obtained by mating of heterozygous mice of mixed genetic background C57BL/6-129/SvJ-CBA. For the immunohistochemical study on PTHrP and type I PTH/PTHrP receptor expression, F1 embryos were obtained from C57BL/6 females mated to CBA males. The embryos were kept on a 14:10 h light-dark rhythm. Gestation was assumed to have begun in the middle of the dark period.

Genotyping of the embryos was performed by PCR. Length of embryos was measured before fixation, after removing the extraembryonic membranes and leaving the embryo intact.

4.3. Immunohistochemistry

Embryos were fixed in 4% buffered paraformaldehyde and embedded in paraffin, except for Fig. 6C,D, where sections used were obtained from embryos which were quick-frozen in tissue freezing medium (Electron Microscopy Center, Washington). Deparaffinized 6 mm sections were treated with 1% H₂O₂ in 40% methanol for 20 min to block endogenous peroxidase activity, and subsequently pretreated with 0.03% pepsin (Sigma) in diluted HCl (pH 2.0) for 8 min at 37°C. Blocking of non-specific binding and all subsequent antibody incubations were performed in 0.5% Boehringer Milk Powder (BMP) dissolved in TNT (100 mM Tris, 150 mM NaCl, 0.05% Tween-20, and 0.04% Triton X-100). Incubation of first antibody was overnight at 4°C. After washing with TNT, sections were incubated with biotinylated secondary antibody (Amersham) for 45 min at 37°C, washed with TNT and incubated with horseradish peroxidase conjugated streptavidin (Amersham) for 30 min at 37°C. Signals were amplified by peroxidase induced deposition of biotinylated tyramides using the signal amplification kit of DuPont. After washing, sections were incubated with horseradish peroxidase conjugated streptavidin for 30 min at 37°C, washed and signals were developed using 3-amino-9-ethylcarbazole (AEC, Sigma). Sections were counterstained using hematoxylin and mounted in Kaisers glycerin.

4.4. Establishment of ES cell lines with a homozygous deletion of the type I PTH/PTHrP receptor gene

ES cell lines were established as previously described (Robertson, 1987). In short, first to introduce a β -galactosidase transgene as marker, C57BL/6 mice heterozygous for the type I PTH/PTHrP receptor knock-out (Lanske et al., 1996) were mated with C57BL/6 mice carrying a β -galactosidase transgene which was engineered to be expressed ubiquitously (Zambrowicz et al., 1997). By further mating, mice heterozygous for the type I PTH/PTHrP receptor knock-out and homozygous for the β -galactosidase transgene were made. They were then mated with mice heterozygous for the PTH/PTHrP receptor knock-out to produce blastocysts. Collected blastocysts were placed onto fibroblasts feeder layers and cultured. When blastocysts hatched and ICMs proliferated, ICMs were isolated from underlying trophoblasts and cultured onto new feeder layers. After about a week, ES cells were identified, isolated and cultured further. From 34 collected blastocysts, 17 new ES cell lines were successfully established. Among them, 13 lines were genotyped by Southern blot. All of them were heterozygous for the β -galactosidase transgene, as expected. Four of them

were homozygous for the type I PTH/PTHrP receptor knock-out, four of them heterozygous, and five of them wild-type. In this study, experiments were performed with the wild-type ES cell line 9(+/+), and the knock out ES cell lines 1(-/-) and 5(-/-).

4.5. Cell culture and transfections

F9 EC cells (ATCC) and ES cells were cultured as described elsewhere (van de Stolpe et al., 1993). Transient transfections were performed using the calcium phosphate precipitation method. One day prior to transfection, the COS-7 or 293 cells were plated at a density of 2×10^4 cells/cm² in six-well tissue culture clusters. The following day cells were transfected with a mouse type I PTH/PTHrP receptor expression vector (cMR13, Karperien et al., 1996) or an empty expression vector.

4.6. Generation and affinity-purification of anti-type I PTH/PTHrP receptor antibodies

Plasmids encoding for GST-fusion protein with either a part of the extracellular domain or the C-terminal intracellular domain of the mouse type I PTH/PTHrP receptor (cMR13), were produced by cloning of, respectively, an *Ava*I restriction fragment coding for amino acid 90–179 and a *Pvu*II restriction fragment coding for amino acid 531–589, into pGEX-3X (Promega). Expression and purification of GST fusion proteins were done as previously described (Smith, 1993).

Antisera were obtained by primary immunization of New Zealand White rabbits via subcutaneous injection of 100 µg GST fusion protein coupled to glutathione-agarose beads in Freund's complete adjuvant, followed by booster immunizations with 100 µg GST fusion protein in Freund's incomplete adjuvant for every 4 weeks. The animals were bled 10 days after each boost. The antibodies A27 and P31, directed against, respectively, the extracellular and intracellular part of the type I PTH/PTHrP receptor, were affinity-purified as previously described (Koff et al., 1992).

4.7. Western blotting

Cells were washed twice with ice-cold PBS, scraped in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mg/ml leupeptin, 20 mg/ml aprotinin and 3 mM phenylmethylsulfonylfluoride), and protein concentrations of the lysates were determined. Preparation of samples for Western blotting on thrombomodulin was done by adding of appropriate amounts of non-reducing sample buffer (Laemmli sample buffer without p-mercaptoethanol), and heating for 5 min at 95°C. Preparation of samples for Western blotting on the type I PTH/PTHrP receptor was done by adding of appropriate amounts of urea saturated non-reducing sample buffer, and shearing of the samples through a

needle. Equal amounts of proteins were loaded on a 10% SDS-polyacrylamide gel blotted to Immobilon-P PVDF membrane, incubated with A27, P31 or anti-thrombomodulin monoclonal antibody 273-34A (Kennel et al., 1987), and subsequently incubated with peroxidase-labeled secondary antibody, followed by ECL. The fold increase in thrombomodulin levels was quantified by computerized densitometry using NIH Image.

4.8. Immunofluorescence

Cells were washed twice with PBS, fixed with 2% paraformaldehyde, and incubated with 20 mM NH₄Cl for 30 min. For detection of type I PTH/PTHrP receptor with antibody A27 or P31, cells were incubated with, respectively, PBS/0.2% BSA for 1 h or treated with 0.1% Triton for 5 min prior to incubation with PBS/0.2% BSA for 1 h. Subsequent antibody incubations were done in PBS/0.2% BSA as well. Detection of thrombomodulin was performed as previously described (Verheijen et al., 1999). Incubations with first antibodies were for 1 h at RT, the cells were rinsed extensively in PBS, and incubated with CY3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT, again extensively rinsed, and mounted in Moviol.

Acknowledgements

We would like to thank Dr. S.J. Kennel for the generous supply of antibodies, Dr. J. Mosely for PTHrP fragments, Dr. H. Juppner for full length hPTH, and Dr. Ir.J.A.J. Faber (Centre of Biostatistics, University Utrecht) and S.C.M. van Wees for help with statistics. Thanks also to J. Heinen and F. Vervoordeldonk for excellent photographic reproductions. This work was supported by grant 90346102 of the Netherlands Organisation for Scientific Research (NWO).

References

- Abou-Samra, A.B., Juppner, H., Force, T., Freeman, M.W., Kong, X.F., Schips, E., Urena, P., Richards, J., Bonventre, J.V., Polts, J.T. Jr., Kronenberg, H.M., Segre, G., 1992. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulated intracellular accumulation of both cAMP and inositol triphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. USA* 89, 2732–2736.
- Behrendtsen, O., Alexander, C.M., Werb, Z., 1995. Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos. *Development* 121, 4137–4148.
- Brown, N.A., 1990. Routine assessment of morphology and growth: scoring systems and measurements of size. In: Copp, A.J., Cockcroft, D.L. (Eds.), *Postimplantation Mammalian Embryos: a Practical Approach*. IRL Press, Oxford, pp. 93–108.
- Chan, S.D.H., Strewler, G.J., King, K.L., Nissenson, R.A., 1990. Expression of a parathyroid hormone-like protein and its receptor during dif-

- ferentiation of embryonal carcinoma cells. *Mol. Endocrinol.* 4, 638–646.
- Cockcroft, D.L., Gardner, R.L., 1987. Clonal analysis of the developmental potential of 6th and 7th day visceral endoderm cells in the mouse. *Development* 101, 143–155.
- Dailifos, A.G., Weir, E.C., Dreyer, B.E., Broadus, A.E., 1992. Stretch induced parathyroid hormone-related peptide gene expression in the rat uterus. *J. Biol. Chem.* 267, 23455–23458.
- Fenton, A.J., Kem, D.B.E., Hammons, R.C., Mitchell, K., Mosely, J.M., Martin, T.J., Nicholson, G.C., 1991. A potent inhibitor of osteoclastic bone resorption within a highly conserved pentapeptide region of PTHrP. *Endocrinology* 129, 3424–3426.
- Fukayama, S., Takigata, A.H., Davis, J.N., Chisholm, J.C., 1995. Signaling by N- and C-terminal sequences of parathyroid hormone-related protein in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* 92, 10182–10186.
- Healy, A.M., Rayburn, H.B., Rosenberg, R.D., Weller, H., 1995. Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. *Proc. Natl. Acad. Sci. USA* 92, 850–854.
- Hebert, T.E., Moffett, S., Morello, J.P., Loisel, T.P., Bisher, D.G., Gorret, C., Bouvier, M., 1996. A peptide derived from a β_2 -adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem.* 271, 16384–16392.
- Hogan, B.L.M., Tilly, R., 1981. Cell interactions and endoderm differentiation in cultured mouse embryos. *J. Embryol. Exp. Morph.* 62, 379–394.
- Hogan, B.L.M., Newman, R., 1984. A scanning electron microscope study of the extraembryonic endoderm of the 8th-day mouse embryo. *Differentiation* 26, 138–143.
- Imada, M., Imada, S., Iwasaki, H., Kume, A., Yamaguchi, H., Moore, E.E., 1987. Fetomodulin: marker surface protein of fetal development which is modulated by cyclic AMP. *Dev. Biol.* 122, 483–491.
- Imanota, N., Akiyama, M., Kubota, N., Juppner, H., 1995. Characterization of a novel PTHrP-receptor with specificity for the carboxyl-terminal region of the PTHrP(1-84). *Endocrinology* 136, 4732–4740.
- Janovick, J.A., Conn, M.P., 1996. Gonadotropin releasing hormone agonist provokes homologous receptor microsegregation: an early event in seven-transmembrane receptor mediated signaling. *Endocrinology* 137, 3602–3605.
- Karaplis, A., Luz, A., Glowacki, J., Bronson, R.T., Tybulewicz, V., Kronenberg, H.M., Mulligan, R.C., 1994. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone related peptide gene. *Genes Dev.* 8, 277–289.
- Karperien, M., van Dijk, T.B., Hocijmakers, S.T., Cremers, F., Abou-Samra, A.B., Boonstra, J., de Laat, S.W., Defize, L.H.K., 1994. Expression pattern of parathyroid hormone-related peptide receptor mRNA in mouse postimplantation embryos indicates involvement in multiple developmental processes. *Mech. Dev.* 47, 29–42.
- Karperien, M., Lanser, P., de Laat, S.W., Boonstra, J., Defize, L.H.K., 1996. Parathyroid hormone-related peptide mRNA expression during postimplantation development evidence for involvement in multiple differentiation processes. *Int. J. Dev. Biol.* 40, 599–608.
- Karpi, D.B., Arnaud, C.D., King, K., Bambino, T., Winer, J., Nyireddy, K., Nussenson, R.A., 1987. The canine renal parathyroid hormone receptor is a glycoprotein: characterization and partial purification. *Biochemistry* 26, 7825–7833.
- Kennel, S.J., Hotchkiss, J.A., Rorvik, M.C., Allison, D.P., Poole, L.J., 1987. Rat monoclonal antibodies to mouse lung components for analysis of fibrosis. *Exp. Mol. Pathol.* 47, 110–124.
- Koff, A., Giordano, A., Desai, D., Yamashita, K., Wade, J., Elledge, S., Nishimoto, T., Morgan, D.O., Franz, B.R., Roberts, J.M., 1992. Formation and activation of a cyclin F-box2 complex during the G1 phase of the human cell cycle. *Nature* 357, 1689–1694.
- Kovacs, C.S., Lanske, B., Hunzelman, J.L., Guo, J., Karanlis, A.C., Kronenberg, H.M., 1996. Parathyroid hormone-related peptide (PTHrP) regulates fetal-placental calcium transport through receptor distinct from the PTH/PTHrP receptor. *Proc. Natl. Acad. Sci. USA* 93, 15233–15238.
- Lanske, B., Karaplis, A.C., Lee, K., Luz, A., Vorkamp, A., Piro, A., Karperien, M., Defize, L.H.K., Ho, C., Mulligan, R.C., Abou-Samra, A.B., Juppner, H., Segre, G.V., Kronenberg, H.M., 1996. PTHrP/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273, 663–666.
- Mallett, L.E., 1991. The parathyroid polypeptides: new concepts in the spectrum of peptide action. *Endocr. Rev.* 12, 110–117.
- Orloff, J.J., Reddy, A., de Papp, A.E., Yang, K.H., Soifer, N.E., Stewart, A.F., 1994. Parathyroid hormone-related protein as a prohormone: post-translational processing and receptor interactions. *Endocr. Rev.* 15, 40–60.
- Orloff, J.J., Kats, Y., Urena, P., Schipani, E., Vasavada, R.C., Philbrick, W.M., Behal, A., Abou-Samra, A.B., Segre, G.V., Juppner, H., 1995. Further evidence for a novel receptor for amino-terminal parathyroid hormone-related protein on keratinocytes and squamous carcinoma cell lines. *Endocrinology* 136, 3016–3023.
- Paspallaris, V., Vargas, S.J., Gillespie, M.T., Williams, F.D., Danks, J.A., Mosely, J.M., Story, M.E., Pennefather, J.N., Leaver, D.D., Martin, T.J., 1992. Oestrogen enhancement of the myometrial response to exogenous parathyroid hormone-related protein (PTHrP), and tissue localization of endogenous PTHrP and its messenger RNA in the virgin rat uterus. *J. Endocrinol.* 134, 415–426.
- Robertson, E.J., 1987. Embryo derived stem cell lines. In: Robertson, E.J. (Ed.), *Teratocarcinomas and Embryonic Stem Cells: a Practical Approach*. IRL Press, pp. 71–112.
- Shigono, C., Hiraki, Y., Westerberg, D.P., Potts, J.T. Jr., Segre, G.V., 1988. Parathyroid hormone receptors are plasma membrane glycoproteins with asparagine-linked oligosaccharides. *J. Biol. Chem.* 263, 3872–3878.
- Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M.Y., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Anazaitis, N., Doetschman, T., 1992. Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 359, 693–699.
- Smith, D.B., 1993. Purification of glutathione S-transferase (fusion proteins). *Methods. Mol. Cell. Biol.* 4, 220–229.
- Strickland, S., Mahdavi, V., 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* 15, 393–403.
- Strickland, S., Smith, K.K., Marotti, K.R., 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* 21, 347–355.
- van de Stolpe, A., Karperien, M., Lowik, C.W.G.M., Juppner, H., Segre, G.V., Abou-Samra, A.B., de Laat, S.W., Defize, L.H.K., 1993. Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell. Biol.* 120, 235–243.
- Thiede, M.A., Harm, S.C., Hasson, D.M., Gardner, R.M., 1991. In vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17 β -estradiol. *Endocrinology* 128, 2317–2323.
- Usdin, T.B., Gruber, C., Bonner, T.I., 1995. Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTHrP receptor. *J. Biol. Chem.* 270, 15455–15458.
- Verheijen, M.H.G., Wolthuis, R.M.F., Bos, J.L., Defize, L.H.K., 1999. The Ras/Ryk pathway induces primitive endoderm but prevents parietal endoderm differentiation of F9 embryonal carcinoma cells. *J. Biol. Chem.*, in press.
- Weiler-Guethler, H., Aird, W.C., Rayburn, H., Husain, M., Rosenberg, R.D., 1996. Developmentally regulated gene expression of thrombospondin in postimplantation mouse embryos. *Development* 122, 2271–2281.
- Williams, E.D., Leaver, D.D., Danks, J.A., Mosely, J.Y.M., Martin, T.J., 1994. Effect of parathyroid hormone-related protein (PTHrP) on the contractility of the myometrium and localization of PTHrP in the uterus of pregnant rats. *J. Reprod. Fertil.* 102, 209–214.

- Yamamoto, S., Morimoto, I., Yanagihara, N., Zeki, K., Fujihira, T., Izumi, F., Yamashita, H., Eto, S., 1997. Parathyroid hormone-related peptide(1-34) [PTHrP(1-34)] induce vasopressin release from the rat supraoptic nucleus in vitro through a novel receptor distinct from a type I or type II PTH/PTHrP receptor. *Endocrinology* 138, 2066–2072.
- Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G., Soriano, P., 1997. Disruption of overlapping transcripts in the ROSA bgeo 26 gene trap strain leads to widespread expression of β -galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. USA* 94, 3789–3794.

Endocrinology

HOME HELP FEEDBACK SUBSCRIPTIONS ARCHIVE SEARCH TABLE OF CONTENTS



Endocrinology, Vol 124, 111-118, Copyright © 1989 by Endocrine Society

ARTICLES

Synthesis of a gene encoding parathyroid hormone-like protein-(1-141): purification and biological characterization of the expressed protein

M Thorikay, S Kramer, FH Reynolds, JM Sorvillo, L Doescher, T Wu, CA Morris, WJ Burtis, KL Insogna and DM Valenzuela

Oncogene Science, Inc., Manhasset, New York 11030.

PTH-like proteins (PTHLP), which are associated with humoral hypercalcemia of malignancy, have recently been purified. Isolation of their corresponding cDNAs has revealed that they are derived from a single gene. In this report a synthetic gene encoding PTHLP-(1-141), a 141-amino acid protein corresponding to the most abundant PTHLP cDNA detected in human tumors, was expressed in bacteria and purified to homogeneity. Recombinant (r) PTHLP-(1-141) migrates with an aberrantly high mol wt on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, presumably as a result of its unusually basic pI. rPTHLP-(1-141), like PTH, induced hypercalcemia in rats, caused release of ⁴⁵Ca from fetal rat bones, and stimulated the synthesis of cAMP by rat osteosarcoma cells and canine renal membrane preparations. A comparison of the abilities of rPTHLP-(1-141) and bovine PTH-(1-34) to stimulate cAMP synthesis indicated rPTHLP-(1-141) to be 5-fold more potent in the osteosarcoma assay, while nearly 30-fold less active in the renal membrane adenylate cyclase assay. Although 100-fold less potent than bovine PTH-(1-34) in promoting bone resorption, rPTHLP-(1-141) was a potent calcemic factor in vivo, inducing a rise in serum calcium from 10.4 to 14.5 mg/dl when infused into rats at 1.3 micrograms/h. These results support previous assumptions that PTHLP is the humoral factor responsible for humoral hypercalcemia of malignancy. In addition, they suggest substantial differences between PTHLP and PTH in the regulation of calcium homeostasis.

This Article

- ▶ [Alert me when this article is cited](#)
- ▶ [Alert me if a correction is posted](#)

Services

- ▶ [Email this article to a friend](#)
- ▶ [Similar articles in this journal](#)
- ▶ [Similar articles in PubMed](#)
- ▶ [Alert me to new issues of the journal](#)
- ▶ [Download to citation manager](#)
- ▶ [Request Copyright Permission](#)

Citing Articles

- ▶ [Citing Articles via Google Scholar](#)

Google Scholar

- ▶ [Articles by Thorikay, M.](#)
- ▶ [Articles by Valenzuela, D. M.](#)
- ▶ [Search for Related Content](#)

PubMed

- ▶ [PubMed Citation](#)
- ▶ [Articles by Thorikay, M.](#)
- ▶ [Articles by Valenzuela, D. M.](#)
- ▶ [PubMed/NCBI databases](#)
 - [Nucleotide](#)
 - [Protein](#)
 - [Compound via MeSH](#)
 - [Substance via MeSH](#)
- ▶ [Hazardous Substances DB](#)
 - [CALCIUM COMPOUNDS](#)
 - [CALCIUM, ELEMENTAL](#)
 - [PARATHYROID HORMONE](#)

APPEAL BRIEF Serial No. 10/520,085

Evidence Appendix (d) - Page 1 of 1

A service of the U.S. National Library of Medicine
and the National Institutes of HealthMy NCBI [?]
[Sign in] [Registered]

All Databases

PubMed

Nucleotide

Protein

Genome

Structure

OMIM

PMC

Journals

Books

Search PubMed

for

Go

Clear

Advanced Search (beta)

Limits

Preview/Index

History

Clipboard

Details

Display

AbstractPlus

Show

20

Sort By

Send to

All: 1

Review: 0

1: *Endocrinology*. 1991 Oct;129(4):1762-8.

Links

A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts.**Fenton AJ, Kemp BE, Kent GN, Moseley JM, Zheng MH, Rowe DJ, Britto JM, Martin TJ, Nicholson GC.**

Department of Medicine, University of Western Australia, Fremantle Hospital.

PTH-related protein (PTHrP) interacts, via its amino-terminal 34 residues, with PTH receptors on osteoblasts to stimulate osteoclastic bone resorption indirectly. We now report that mature hPTHrP-(1-141) (EC50, approximately 10⁻¹¹ M) and a carboxyl-terminal fragment, PTHrP-(107-139) (EC50, approximately 10⁻¹⁵ M), are potent inhibitors of resorption in an isolated rat osteoclast bone resorption assay, whereas hPTHrP-(1-108) and hPTHrP-(1-34) are inactive in this respect. PTHrP-(107-139) also inhibits resorption in a rat long bone organ culture system and reduces osteoclast spreading. PTHrP-(107-139) does not act on osteoclasts via a cAMP signal transduction mechanism, but its effects may be mediated by protein kinase-C. This previously unrecognized action of PTHrP, to inhibit osteoclastic bone resorption directly, indicates that PTHrP may be a precursor of multiple biologically active peptides with differing physiological functions.

PMID: 1915066 [PubMed - indexed for MEDLINE]

Display

AbstractPlus

Show

20

Sort By

Send to

Related Articles

Long term culture of disaggregated rat osteoclasts: inhibition of bone resorption and reduction of osteoclast-like cell number by calcitonin and PTHrP(30-136). [1993]

Human parathyroid hormone-related peptide (107-111) does not inhibit bone resorption in neonatal mouse calvariae. [Endocrinology. 1992]

Carboxyl-terminal peptides from parathyroid hormone-related protein stimulate osteoclast-like cells. [Endocrinology. 1993]

Review Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl-terminal ligands. [Endocr Rev. 2003]

Review [Parathyroid hormone related peptide (PTHrP) and bone metabolism] [Arch Physiol Biochem. 1995]

a See Reviews... | > See All...

Write to the Help Desk

NCBI | NLM | NIH

Department of Health & Human Services

Privacy Statement | Freedom of Information Act | Disclaimer

Up-regulation of parathyroid hormone-related protein in folic acid-induced acute renal failure

SOLEDAD SANTOS, RICARDO J. BOSCH, ARANTXA ORTEGA, RAQUEL LARGO, TERESA FERNÁNDEZ-AGULLÓ, ROSA GAZAPO, JESÚS EGIDO, and PEDRO ESRIT

Bone and Mineral Metabolism Laboratory, Renal Research Laboratory, and Biochemistry Department, Fundación Jiménez Díaz and Autónoma University, Madrid; and Laboratory of Renal Physiology and Experimental Nephrology, Department of Physiology, Alcalá University, Alcalá de Henares, Spain

Up-regulation of parathyroid hormone-related protein in folic acid-induced acute renal failure.

Background. Parathyroid hormone (PTH)-related protein (PTHrP) is present in many normal tissues, including the kidney. Current evidence supports that PTHrP is involved in renal pathophysiology, although its role on the mechanisms of renal damage and/or repair is unclear. Our present study examined the changes in PTHrP and the PTH/PTHrP receptor (type 1) in folic acid-induced acute renal failure in rats. The possible role of PTHrP on the process of renal regeneration following folic acid administration, and potential interaction between angiotensin II (Ang II) and endothelin-1, and PTHrP, were examined in this animal model.

Methods. PTHrP, PTH/PTHrP receptor, ACE, and prepro-endothelin-1 (preproET-1) mRNA levels in the rat kidney were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and/or RNase protection assay. Immunohistochemistry also was performed for PTHrP, the PTH/PTHrP receptor, and Ang II in the renal tissue of folic acid-injected rats. The role of PTHrP on tubular cell proliferation following folic acid injury was investigated in vitro in rat renal epithelial cells (NRK 52E). PTHrP secretion in the medium conditioned by these cells was measured by an immunoradiometric assay specific for the 1-36 sequence.

Results. Using RT-PCR, PTHrP mRNA was rapidly (1 hour) and maximally increased (3-fold) in the rat kidney after folic acid, decreasing after six hours. At 72 hours, renal function was maximally decreased in these rats, associated with an increased PTHrP immunostaining in both renal tubules and glomeruli. In contrast, the PTH/PTHrP receptor mRNA (RNase protection assay) decreased shortly after folic acid administration. Moreover, PTH/PTHrP receptor immunostaining dramatically decreased in renal tubular cell membranes after folic acid. A single subcutaneous administration of PTHrP (1-36), 3 or 50 µg/kg body weight, shortly after folic acid injection increased the number of tubular cells staining for proliferating cell nuclear antigen by 30% ($P < 0.05$) or 50% ($P < 0.01$), respectively,

in these rats at 24 hours, without significant changes in either renal function or calcemia. On the other hand, this peptide failed to modify the increase (2-fold over control) in ACE mRNA, associated with a prominent Ang II staining into tubular cell nuclei, in the kidney of folic acid-treated rats at this time period. The addition of 10 mmol/L folic acid to NRK 52E cells caused a twofold increase in PTHrP mRNA at six hours, without significant changes in the PTH/PTHrP receptor mRNA. The presence of two anti-PTHrP antibodies, with or without folic acid, in the cell-conditioned medium decreased (40%, $P < 0.01$) cell growth.

Conclusions. Renal PTHrP was rapidly and transiently increased in rats with folic acid-induced acute renal failure, featuring as an early response gene. In addition, changes in ACE and Ang II expression were also found in these animals. PTHrP induces a mitogenic response in folic acid-damaged renal tubular cells both in vivo and in vitro. Our results support the notion that PTHrP up-regulation participates in the regenerative process in this model of acute renal failure and is a common event associated with the mechanisms of renal injury and repair.

Parathyroid hormone (PTH)-related protein (PTHrP) is present in the vast majority of normal fetal and adult tissues, including the kidney, and it appears to act as an autocrine/paracrine factor [1, 2]. In adults, PTHrP is widely present along the nephron and in the intrarenal arterial tree, and current evidence points to an emerging role of PTHrP in renal physiology [1, 3, 4]. An increasing number of studies support the notion that PTHrP, acting through its N-terminal region homologous to PTH, is involved in the control of vascular tone [5, 6]. In this regard, PTHrP (1-34) induces vasorelaxation in the pre-constricted isolated rabbit kidney [3]. Moreover, this peptide increases glomerular filtration rate and renal blood flow when infused into the renal artery of anesthetized rats [7]. However, the true role of local PTHrP in the kidney as a regulator of the renal vascular tone remains unclear, since this peptide also increases renal renin secretion [8]. Recent studies have demonstrated that PTHrP might directly modulate glomerular function

Key words: kidney regeneration, angiotensin converting enzyme, endothelin-1, angiotensin II, mitogenesis, renal repair, tissue repair.

Received for publication August 23, 2000

and in revised form March 15, 2001

Accepted for publication March 29, 2001

© 2001 by the International Society of Nephrology

by interacting with the contracting-relaxing capabilities of glomerular mesangial cells [9, 10].

Parathyroid hormone-related protein also exhibits growth-regulatory properties in renal tubular and mesangial cells [4, 10–13]. Renal PTHrP expression is rapidly and transiently up-regulated during the recovery period after ischemic injury *in vivo* and *in vitro* and in cyclosporine-induced chronic nephrotoxicity [4, 13, 14]. Moreover, we recently found a sequential increase of PTHrP, associated with a decreased PTH/PTHrP (so called type 1) receptor, in the renal cortex of rats during the development of proteinuria after albumin overloading [15]. These findings suggest that PTHrP overexpression might be related to the mechanisms associated with renal injury and also with the progression of renal damage.

In the present study, considering the PTHrP features in glomerular function and tubular growth described previously in this article, we hypothesized that this protein might play a role in the injured kidney and in the subsequent renal regenerative process. To assess this hypothesis, the renal expression of PTHrP and the PTH/PTHrP receptor were examined in rats with acute renal failure induced by folic acid. A dramatic renal tubular hyperplasia occurs in this animal model, but in contrast to other experimental models of acute renal failure, kidney lesions following folic acid injection are only moderate [16–18]. Since the mitogenic effects of PTHrP on renal cells had only been characterized *in vitro*, we investigated whether exogenous administration of PTHrP would increase renal epithelial regeneration in this *in vivo* model. In addition, the changes of angiotensin-converting enzyme (ACE) and preproendothelin-1 (preproET-1), whose expression increase in parallel to PTHrP in the renal cortex of rats with intense proteinuria [15], as well as those of angiotensin II (Ang II) were evaluated in the kidney of rats after folic acid injury. In addition, the putative direct effects of folic acid on PTHrP production in rat kidney epithelial cells *in vitro* and its role on cell growth were assessed. Our studies provide further insights into the emerging role of PTHrP as a renal regulating factor [19], supporting its participation in the regenerative process in the damaged kidney.

METHODS

Animal model

Male Wistar rats (250 g body weight) were fed standard rat chow ad libitum and were given free access to water. One group of rats received a single intraperitoneal injection of folic acid (Sigma, St. Louis, MO, USA), 250 mg/kg in 150 mmol/L sodium bicarbonate (vehicle). Control animals received the same volume of vehicle alone. At different time periods up to 10 days after folic acid injection, four to eight animals from each group were sacrificed under ether anesthesia, and kidneys were

removed. At 1.5 hours following folic acid administration, other groups of six animals each were injected subcutaneously with PTHrP (1–36) (kindly donated by Dr. A.F. Stewart, Department of Endocrinology, University of Pittsburgh, PA, USA), at 0.3, 3, and 50 μ g/kg, respectively, in saline with 0.1% bovine serum albumin (BSA). These doses were selected because they have shown to elicit various renal and bone effects in humans and rats [20, 21]. On the other hand, acute or chronic administration of this peptide and other related peptides in this dose range into normal subjects failed to affect either renal function or calcemia [20, 21]. At 24 hours after folic acid administration, these rats were killed under ether anesthesia, and kidneys were collected. One kidney from each animal in all groups was fixed in 4% buffered p-formaldehyde for light microscopy examination and immunohistochemistry. The other kidney was snap-frozen in liquid nitrogen and stored at -70°C for subsequent RNA extraction. Some kidney tissue samples were freeze-dried, and then lyophilized samples were dissolved in 0.1 N NaOH. Protein was determined in tissue extracts by the Bradford's method, using BSA as the standard [22].

Blood chemistry

Blood was taken by cardiac puncture under ether anesthesia after an overnight fast. Plasma creatinine and blood urea nitrogen (BUN) were determined by auto-analyzer (Hitachi Chemistry System; Boehringer Mannheim, Mannheim, Germany).

Histologic studies and immunohistochemistry

Fixed renal tissue sections were dehydrated by graded ethanols and xylene and then embedded in paraffin. Paraffin-embedded tissue sections (4 μ m) were mounted on 3-aminopropyltriethoxy silane-treated slides for histologic evaluation or immunohistochemistry. Kidney sections were stained with hematoxylin and eosin.

Parathyroid hormone-related protein immunostaining was carried out with affinity purified anti-PTHrP antibody Ab-2 (Oncogene, Uniondale, NY, USA), recognizing the sequence 34 to 53 of human and rat PTHrP [14, 15]. The PTH/PTHrP receptor staining was performed with an affinity-purified antibody against the extracellular domain of this receptor in the rat (Ab VII; Babco, Richmond, CA, USA) [23]. For Ang II staining, a rabbit antiserum to Ang II (Peninsula Laboratories, San Carlos, CA, USA) was used. Proliferative activity was evaluated with a monoclonal antiproliferating cell nuclear antigen (PCNA; Clone PC10; Dako, Glostrup, Denmark). Immunostaining was performed as described in detail [15, 23]. Briefly, tissue sections were incubated with 1.5% normal goat serum in phosphate-buffered saline (PBS) for 30 minutes and then with 4 μ g/mL of Ab-2 antibody, 5 μ g/mL of Ab-VII antibody, 400-fold dilution of anti-Ang II anti-

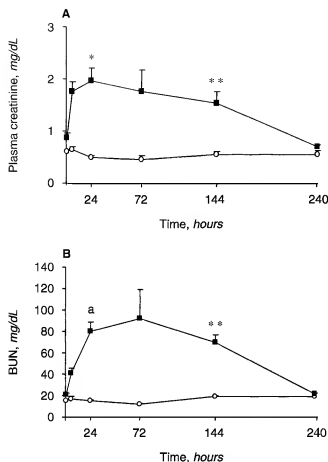


Fig. 1. Time-course of changes in plasma creatinine (A) and blood urea nitrogen (BUN; B) in rats with folic acid-induced acute renal failure. Measurements were from blood samples obtained in rats after overnight fast, using standard methods. Points are mean \pm SEM of eight animals at each time period. All values were significantly different ($P < 0.05$ or less) from the corresponding values in vehicle-injected (control) rats (○) at each time period studied within one hour and six days. * $P < 0.05$; ^a $P < 0.01$, compared with the corresponding values in folic acid-treated rats (■) at six hours. ** $P < 0.05$ compared with 240 hours.

serum, or 150-fold dilution of PCNA antibody for either two hours (Ab-2 and Ab-VII) or 30 minutes (Ang II and PCNA antibodies). Sections were subsequently incubated with either goat anti-rabbit (Ab-2 and Ab-VII) or anti-mouse IgG (PCNA antibody) and the avidin-biotin-peroxidase complex (Vector, Burlingame, CA, USA). Following incubation with the Ang II antiserum, the sections were incubated with a polymer-peroxidase complex (Envision+System; Dako). 3,3'-Diaminobenzidine was used as the chromogen. Some tissue sections were treated either with nonimmunogenic IgG or without the primary antibody, as negative controls. Staining of the capillary network and peritubular connective tissue was considered as a positive internal control for Ang II [24]. The tissue sections were counterstained with Carazzi's hematoxylin.

In all cases, morphologic and immunologic evaluations of tissue samples were performed by two independent observers in a blinded fashion. PTHrP immunostaining was scored at glomerular and tubular level and graded as follows: 0, negative staining; 1, mild staining; 2, moderate staining; and 3, intense staining. Tubular staining for PCNA was evaluated in three $\times 100$ microscopic fields by counting the number of stained cell nuclei per field and then calculating the mean from each rat. In addition, the number of stained cells per 20 glomeruli without selection was also counted for each rat [25]. The final score was always the mean of the two evaluations.

Cell culture

Rat kidney cells NRK 52E (ATCC CRL 1571), displaying a tubuloeplithelial phenotype [26, 27], were grown in Dulbecco's modified essential medium (DMEM) with 5% fetal bovine serum (FBS; BioWhittaker, Verviers, Belgium), 100 U/mL penicillin, and 100 μ g/mL streptomycin, in 5% CO₂ at 37°C. Cells were serum-depleted for 24 hours before addition of 10 mmol/L folic acid dissolved in 300 mmol/L NaHCO₃. This addition did not change the medium pH. In some experiments, subconfluent cells were incubated for 48 hours in serum-free medium with folic acid in the presence or absence of affinity-purified anti-PTHrP antiserum C7 or C13, recognizing either the C- or N-terminal region of PTHrP, respectively [28]. Then cells were trypsinized and resuspended in trypan blue. Viable cells were counted in a hemocytometer.

RNA extraction

Total RNA was isolated from either rat kidney homogenates obtained with a glass-Teflon homogenizer, or FBS-depleted NRK 52E cells, using TriReagent (1 mL per g wet tissue or 10⁶ cells, respectively; Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

To assess changes in mRNA levels by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA from either rat kidney (5 ng for the PTH/PTHrP receptor; 10 ng for c-fos; and 100 ng for the other genes studied), or NRK 52E (100 ng for the PTH/PTHrP receptor and 20 ng for PTHrP) was reverse transcribed, and resulting cDNA was amplified, using a commercial kit (Access RT-PCR System[®]; Promega, Madison, WI, USA), with specific primers for the rat PTH/PTHrP receptor [23], PTHrP, ACE, preproET-1 [15], and c-fos [5'-GGGAATTCGGAGATCCGAA GGGAAGG-3' (sense), and 5'-CCGGATCCGTGA AGGCTCTCAGACTC-3' (antisense)]. These primers yield PCR amplification products of 266 bp (PTHrP), 495 bp (PTH/PTHrP receptor), 317 bp (ACE), 409 bp (preproET-1), and 316 bp (c-fos). The housekeeping gene,

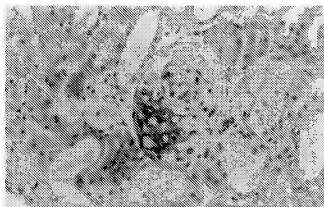


Fig. 2. Histologic examination of a rat kidney section at six hours after folic acid injection. Folic acid deposit (yellow-brownish crystals) is shown in the glomerular mesangium and the glomerular capillary tufts. Tubular dilation was evident, as well as edema and yellowish casts in the tubular epithelium (magnification $\times 200$). Reproduction of this figure in color was supported by the Spanish Ministry of Science and Technology.

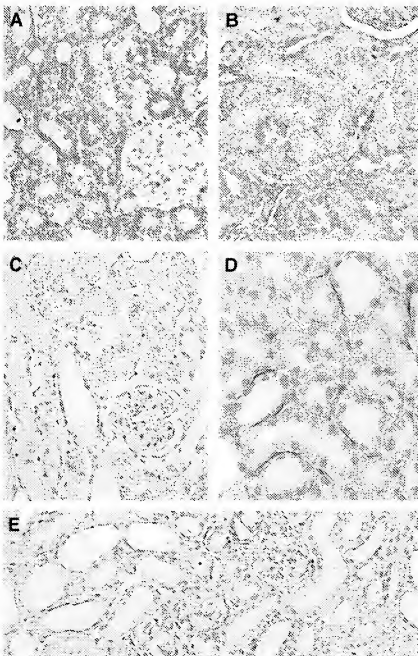


Fig. 4. Immunostaining for parathyroid hormone-related protein (PTHrP) and the PTH/PTHrP receptor in the kidney of folic acid-injured rats. Kidney sections of representative folic acid-treated (A and C) or -untreated (B and D) rats, showing immunolocalization of PTHrP by Ab-2 antibody (A and B), and of the PTH/PTHrP receptor by using Ab-VII antibody (C and D). A dramatic increase in PTHrP positivity was observed in both proximal and distal tubules in folic acid-treated rats. In contrast, PTH/PTHrP receptor staining, which was present in the basolateral membranes of the proximal tubules in the kidney cortex of control rats, almost disappeared at 72 hours after folic acid injection. Negative control without primary antibody (E). Magnifications $\times 100$ (A-C and E) and $\times 500$ (D). Reproduction of this figure in color was supported by the Spanish Ministry of Science and Technology.

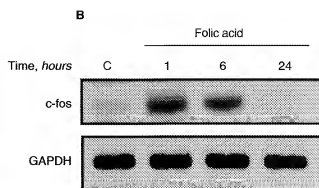
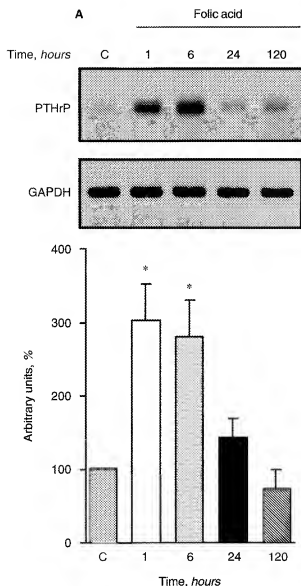


Fig. 3. Parathyroid hormone-related protein (PTHrP) and c-fos mRNA in the rat kidney following folic acid injury. Total RNA was isolated from the kidney of folic acid-treated or -untreated rats at different time periods. (A) PTHrP and GAPDH mRNA, a constitutive control, and (B) c-fos mRNA were evaluated by RT-PCR. (A) Densitometric analysis corresponding to changes in PTHrP mRNA levels, corrected to those of GAPDH mRNA, induced by folic acid. Results are mean \pm SEM corresponding to two different total RNA pools, each obtained from five different animals, and three individual animals per group. * $P < 0.01$ compared with control (C; 100%).

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified using specific primers as a constitutive control [15]. The reaction mixture (10 μ L), with 0.5 μ Ci [α - 32 P]dCTP (3000 Ci/mmol; NEN Life Science Products, Zaventem, Belgium), was incubated for 45 minutes at 48°C and two minutes at 95°C, followed by 30 cycles of one minute at 95°C, one minute at 60°C, and two minutes at 68°C, with a final extension of seven minutes at 68°C. Preliminary experiments established that these conditions provided a linear cDNA amplification in each case. 32 P-labeled PCR products were separated on 4% polyacrylamide gels, which were dried and exposed to autoradiographic film. Values obtained after densitometric scanning (ImageQuant; Molecular Dynamics, Sunnyvale, CA, USA) of the different PCR products were normalized against those of the corresponding GAPDH product.

RNase protection assay

RNase protection analysis was performed as described by Thiede et al [29], using 40 μ g of total RNA and a 481 bp antisense cRNA probe, corresponding to a 151 to 631 bp fragment of the coding region of the rat PTHrP/PTHrP receptor gene, cloned into the PCR-II plasmid (Invitrogen, Groningen, The Netherlands). An antisense cRNA probe corresponding to a 115 bp fragment of the human 28S rRNA gene (Ambion, Austin, TX, USA) was used as an internal standard. Antisense cRNA probes were 32 P-labeled and hybridized to total RNA from each sample in 80% formamide, 40 mmol/L piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.7, and 400 mmol/L NaCl, at 55°C overnight. Samples were sequentially treated with RNase A (40 μ g/mL), RNase T1 (2 μ g/mL), and proteinase K (60 μ g/mL). RNA hybrids were then phe-

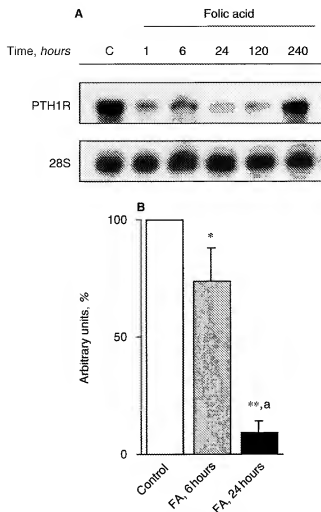


Fig. 5. PTHrP receptor in the rat kidney following folic acid injury. Total RNA was isolated from the kidney of folic acid-treated or -untreated rats at different time periods. (A) Autoradiogram showing the analysis of the PTHrP receptor mRNA, and 28S mRNA as a constitutive control, using RNase protection assay. (B) Densitometric analysis of changes in the PTHrP receptor mRNA levels, corrected to those of 28S mRNA, induced by folic acid. Results are mean \pm SEM corresponding to three different total RNA pools each obtained from five different animals per group. * $P < 0.05$; ** $P < 0.01$ compared with control (100%); * $P < 0.02$ compared with the value at six hours. FA, folic acid; C, control.

nal/chloroform extracted, followed by ethanol precipitation. Protected fragments were fractionated on 6.5% polyacrilamide/7 mol/L urea sequencing gels and analyzed by autoradiography. Values obtained after densitometric scanning of the PTHrP receptor bands were normalized against those of the corresponding 28S band.

PTHrP assay

Parathyroid hormone-related protein in rat plasma and NRK 52E cell-conditioned medium was measured by an immunoradiometric assay, using affinity-purified rabbit antisera against rat and human PTHrP (1-36) as signal and capture antibodies, with a sensitivity of 1 pmol/L [30].

The cell-conditioned medium was freeze dried and then tenfold concentrated in assay buffer before assay.

Statistical analysis

Results are expressed as mean \pm SEM throughout the text. Statistical analysis was performed by either the Kruskal-Wallis test or Mann-Whitney test, when appropriate. $P < 0.05$ was considered significant.

RESULTS

Changes in renal function after folic acid injection

The mean values for plasma creatinine and BUN in folic acid-injected animals were significantly increased at one hour, peaked at 24 hours, and plateaued up to day 6, decreasing thereafter, as compared with the corresponding mean values in vehicle-treated animals (Fig. 1). At day 10 after folic acid administration, rat plasma creatinine and BUN levels reached the corresponding values in the control rats (Fig. 1).

Changes in renal morphology after folic acid-induced acute injury

Histological examination of rat kidney tissue samples indicates that at one hour after folic acid injection, the renal cortex showed tubular distention, and folic acid precipitation presented as yellowish casts in the renal medulla. At six hours following folic acid administration, tubular dilation was a consistent finding, and edema and yellowish casts also were observed in the proximal tubules. At this time period, the tubular epithelium began to become thinner, showing cells coming off from the basal membrane into the lumen, and even some tubules were fragmented (Fig. 2). In addition, occasionally at this time period, folic acid deposition was evident in the glomerular mesangium. Moreover, yellowish casts were present in both the urinary space and the glomerular capillary tufts, which were clearly dilated (Fig. 2). At 24 hours, folic acid deposits were only present in tubules of the renal medulla, while no such deposits could be observed at 48 hours. Ten days after folic acid-induced injury, some tissue disorganization, including edema and tubular dilation, was still evident.

Increase of renal PTHrP expression following folic acid injury

Using semiquantitative RT-PCR, we found that PTHrP mRNA in the rat kidney was rapidly (1 hour) and maximally increased after folic acid injection, being threefold over that of vehicle-treated rats, decreasing after six hours (Fig. 3A). This response pattern was similar to that observed with the protooncogene *c-fos* mRNA in these animals (Fig. 3B).

Furthermore, immunohistochemistry was performed to assess the sites of PTHrP changes in the rat kidney.

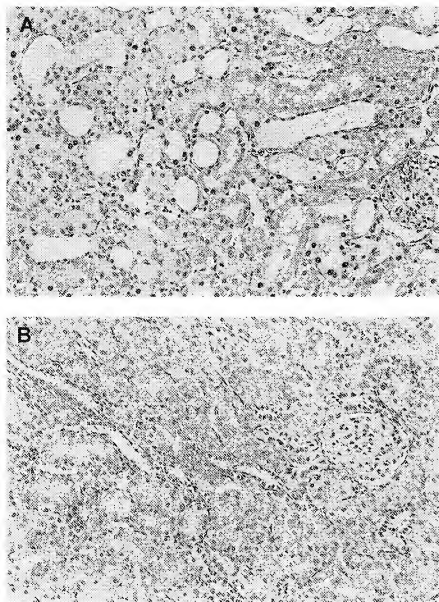


Fig. 6. Renal tubular cell proliferation following folic acid injury. Tubular cell proliferation in the kidney of folic acid-treated and -untreated rats at different time periods was evaluated by immunohistochemistry using a PCNA antibody. Staining in tubule cell nuclei in either a folic acid-treated (A) or a vehicle-treated rat (B) at 24 hours. Magnification $\times 100$.

At 72 hours after folic acid treatment, when renal function was maximally decreased, an increased PTHrP staining in the renal cortex was found (Fig. 4 A, B). The corresponding score values in the renal tubules and glomeruli were as follows: 2.5 ± 0.2 and 1.7 ± 0.1 compared with control rats (1.5 ± 0.1 and 1.0 ± 0.1 , respectively, $P = 0.03$); meanwhile, at day 10, when renal function had normalized, these score values were not different from those in the controls (1.9 ± 0.2 and 0.9 ± 0.3 , respectively, $N = 5$).

The renal PTH/PTHrP receptor decreases after folic acid injury

The PTH/PTHrP receptor mRNA was found to decrease in association with the decrease in renal function

in the rat kidney after folic acid administration, according to the RNase protection assay (Fig. 5) and RT-PCR (data not shown). Furthermore, PTH/PTHrP receptor immunostaining, which was present in both basolateral and luminal membranes in the renal cortical tubules of control rats, dramatically decreased at 72 hours following folic acid treatment (Fig. 4 C, D).

PTHrP increases renal tubular cell proliferation following folic acid injury

Folic acid was found to induce an increase in the number of proliferating renal tubular cells, which was dramatic between 24 and 72 hours and no longer present at 10 days following folic acid injection (Fig. 6). This tubular cell hyperplasia was not accompanied by an in-

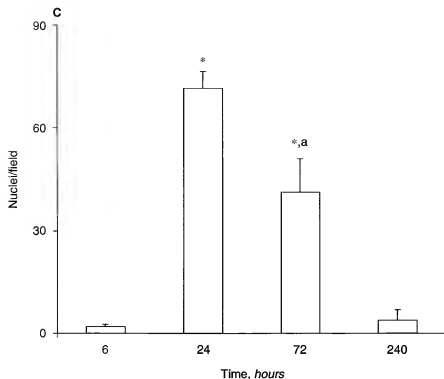


Fig. 6 (Continued). (C) Score values are mean \pm SEM of four to six animals at each time period. * $P < 0.025$ compared with value at six hours. * $P < 0.05$ compared with value at 240 hours.

Table 1. Plasma immunoreactive PTHrP (1-36) at 30 minutes after subcutaneous injection of PTHrP (1-36)

Condition	$\mu\text{g/kg}$ body weight	Concentration pmol/L
FA		<1.2
FA + PTHrP	50	27 ± 10
FA + PTHrP	3	30 ± 9
FA + PTHrP	0.3	10 ± 2

Data are mean \pm SEM of 6–8 rats. Abbreviations are: PTHrP, parathyroid hormone-related protein; FA, folic acid.

creased renal hypertrophy. Thus, the protein content of the rat kidney at 24 and 72 hours after folic acid treatment was (g/g), 0.6 ± 0.1 , and 0.7 ± 0.1 compared with 0.5 ± 0.1 (control, $N = 5$), respectively.

To investigate the relationship between the increase of PTHrP in the rat kidney and renal hyperplasia induced by folic acid, PTHrP (1-36) was administered into rats shortly after folic acid injection. This peptide, at the doses used, was rapidly absorbed and present in rat plasma after its subcutaneous injection (Table 1), consistent with previous findings in humans [20]. A single administration of either 3 or 50 $\mu\text{g/kg}$ body weight, but not 0.3 $\mu\text{g/kg}$, of PTHrP (1-36) into these animals significantly increased the number of PCNA-positive tubular cells at 24 hours after folic acid injection (Fig. 7A). In contrast, at this time period, PTHrP (1-36) administration did not significantly increase PCNA immunostaining in the glomerulus in these animals: 0.8 ± 0.4 and 0.6 ± 0.3 positive cells per glomerulus in folic acid-injected rats, untreated or treated

with 50 $\mu\text{g/kg}$ PTHrP (1-36), respectively ($N = 5$). This effect of exogenously administered PTHrP (1-36) on tubular proliferation was associated with a significant decrease in PTHrP mRNA (Fig. 7B). On the other hand, this mitogenic effect of PTHrP (1-36) was not associated with significant changes in either plasma calcium (data not shown) or renal function (Table 2). Moreover, no significant correlation was found between tubular cell proliferation and either plasma creatinine or BUN at 24 hours following folic acid injection and PTHrP (1-36) (3 or 50 $\mu\text{g/kg}$) treatment [$r = 0.30$, $P = 0.229$ (plasma creatinine) or $r = 0.23$, $P = 0.328$ (BUN), $N = 20$].

Renal expression of ACE and preproET-1 in rats with folic acid injury

Angiotensin-converting enzyme mRNA increased twofold in the kidney of folic acid-injected rats at 24 hours, when renal function was maximally decreased, compared with vehicle-treated controls (Fig. 8). Exogenous administration of PTHrP (1-36), in contrast to its tubular proliferative effect, was not shown to affect this increased ACE mRNA significantly within the same time frame in these animals (Fig. 8). On the other hand, preproET-1 mRNA did not change after either folic acid or PTHrP (1-36) treatments in these rats (Fig. 8A).

Ang II immunoreactivity in the kidney of folic acid-injured rats

Ang II immunoreactivity was also localized in the rat kidney before and after folic acid injection. In vehicle-

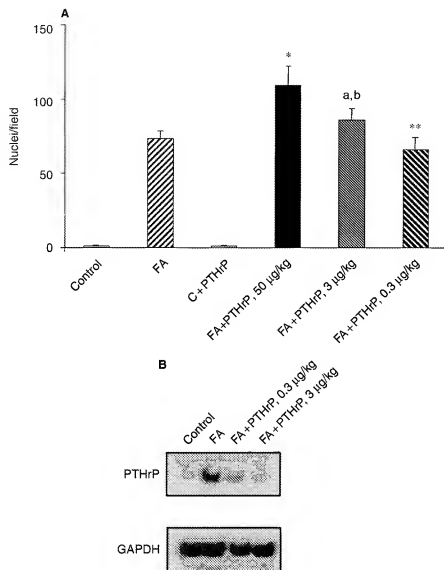


Fig. 7. Effect of PTHrP(1-36) administration on tubular cell proliferation and PTHrP mRNA in the rat kidney after folic acid injury. Different doses of PTHrP (1-36) were administered subcutaneously into rats 1.5 hours after folic acid or vehicle injection. Twenty-four hours later, tubular cell proliferation was assessed by PCNA staining, and PTHrP mRNA was analyzed by RT-PCR. (A) Staining score values are mean \pm SEM of six rats. * $P < 0.01$; $^aP < 0.05$ compared with FA alone. $^bP < 0.05$; $^{**}P < 0.05$, compared with values corresponding to FA and either PTHrP (1-36) at 0.3 or 50 $\mu\text{g}/\text{kg}$ body wt, respectively. (B) Representative results of two different pools of total RNA from three different animals in each case. GAPDH mRNA is shown as a constitutive control. FA, folic acid.

Table 2. Plasma creatinine and blood urea nitrogen (BUN) in vehicle (control) and folic acid (FA)-treated rats, with or without parathyroid hormone-related protein (PTHrP) (1-36), at 24 hours

Condition	$\mu\text{g}/\text{kg}$ body wt	Creatinine	BUN
		mg/dL	
Control		0.5 ± 0.03	15 ± 1
FA		2.0 ± 0.2^a	80 ± 9^a
Control + PTHrP	50	0.5 ± 0.01	13 ± 1
FA + PTHrP	50	2.5 ± 0.3^b	96 ± 7^b
FA + PTHrP	3	2.4 ± 0.4^b	111 ± 8^b
FA + PTHrP	0.3	1.7 ± 0.3^b	105 ± 15^b

Data represent mean \pm SEM of 6 animals per group.

$^aP < 0.01$, relative to control value

$^bP < 0.01$, relative to control + PTHrP value

injected animals, Ang II immunostaining was found in the cytoplasm of tubular cells, which was prominent in the brush border (Fig. 9B). At 24 hours following folic acid injection, intense Ang II positivity was mainly localized into tubular cell nuclei (Fig. 9C).

Role of PTHrP on folic acid-induced injury in renal epithelial cells

To explore the mitogenic effect of PTHrP on renal tubular cells in folic acid-treated rats further, we used the rat renal epithelial cells, NRK 52E. These cells express PTHrP and the PTH/PTHrP receptor (Fig. 10A). Treatment of these cells with 10 mmol/L folic acid stimulated PTHrP mRNA expression, representing twofold over that of vehicle-treated cells at six hours (Fig. 10). In addition, PTHrP immunoreactivity in the cell-conditioned medium in the presence of folic acid for 24 hours

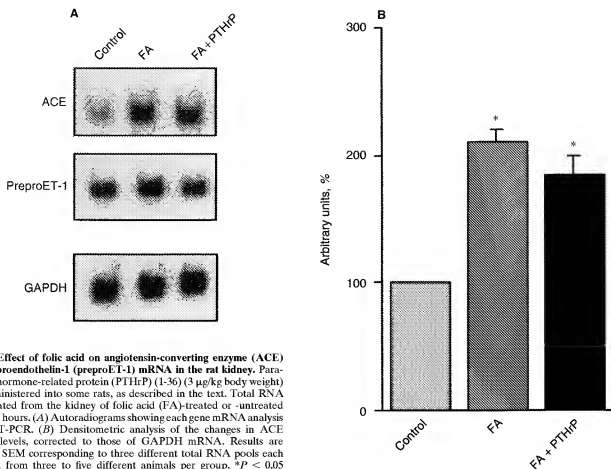


Fig. 8. Effect of folic acid on angiotensin-converting enzyme (ACE) and preendothelin-1 (preproET-1) mRNA in the rat kidney. Parathyroid hormone-related protein (PTHrP) (1-36) (3 μ g/kg body weight) was administered into some rats, as described in the text. Total RNA was isolated from the kidney of folic acid (FA)-treated or -untreated rats at 24 hours. (A) Autoradiograms showing each gene mRNA analysis using RT-PCR. (B) Densitometric analysis of the changes in ACE mRNA levels, corrected to those of GAPDH mRNA. Results are mean \pm SEM corresponding to three different total RNA pools each obtained from three to five different animals per group. * $P < 0.05$ compared with control (100%).

was 0.9 ± 0.2 fmol/mg cell protein, while it was undetectable in vehicle-treated medium for the same time period. On the other hand, 10 mmol/L folic acid failed to affect the PTH/PTHrP receptor mRNA within the same time frame (Fig. 10A).

The presence of two different anti-PTHrP antibodies in the cell-conditioned medium significantly reduced the cell number after 48 hours (Fig. 11). Moreover, the addition of 10 mmol/L folic acid to NRK 52E cells for the same time period induced a decrease in the cell number, which was significantly lower in the presence of the anti-PTHrP antibodies but not nonimmune rabbit IgG (Fig. 11).

DISCUSSION

Parathyroid hormone-related protein and/or the PTH/PTHrP receptor are present in the adult kidney in various nephron sites, including proximal, distal, and collecting tubules, the glomerular podocytes, and also in the intrarenal arterial tree and the macula densa [1, 3, 4, 31, 32]. In the kidney, PTHrP appears to affect glomerular function by both its direct interaction with the contracting-relaxing capabilities of glomerular mesangial cells and

through its potent vasodilatory effect [3, 7, 9, 10]. In addition, *in vitro* studies have demonstrated that this factor is mitogenic for various renal cells, including mesangial cells, distal tubule-like cells, and proximal tubule cells [4, 10–13]. Renal PTHrP expression increases *in vivo* in rats during the recovery phase after ischemic injury and also in rats with chronic renal failure [4, 14, 15].

The recovery of renal function after nephrotoxic or ischemic acute renal failure requires the repair of the damaged renal tubular epithelium, which is apparently dependent on the local production of several growth factors and cytokines [33, 34]. Recent *in vitro* findings suggest an emerging role for PTHrP in the process of renal regeneration following renal ischemia [13]. The present study evaluated the possible involvement of PTHrP in the intense renal tubular hyperplasia following acute renal injury induced by folic acid [16–18]. PTHrP was rapidly and transiently increased in the kidney of folic acid-injected rats. Moreover, PTHrP immunostaining increased in both tubules and glomeruli, associated with the maximal decrease of renal function following folic acid injection. In the renal ischemia model, using

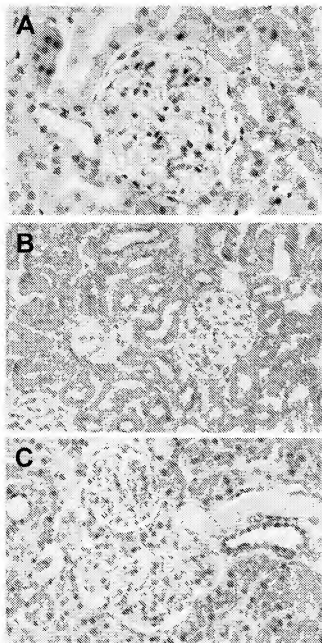


Fig. 9. Immunostaining for angiotensin II (Ang II) in the kidney of folic acid (FA)-injured rats. Kidney sections of representative FA-treated (C) or untreated (A and B) rats, showing Ang II immunocalculation (B and C). Angiotensin II staining was mostly present in the luminal membranes of cortical tubules in the kidney cortex of control rats (B). In contrast, Ang II positivity was predominantly associated with renal tubule cell nuclei in the kidney of FA-treated rats (C). Similar findings were observed in at least four different animals per group. Negative control without primary antibody (A). Magnifications $\times 200$ (A) and $\times 100$ (B and C).

an anti-PTHrP antibody with a similar specificity to that of the antibody used herein, a dramatic increase in tubular, but not in glomerular, PTHrP staining was found in the rat kidney during the recovery phase after ischemia

[4]. These different results regarding glomerular PTHrP staining in both models of acute renal injury might be explained by differences in either antibody affinities or the immunohistochemical technique used in both studies. However, these findings also suggest that different underlying mechanisms mediate the glomerular response in these two animal models of acute renal damage. In this regard, putative glomerular damage associated with the observed folic acid deposits in the glomerulus could be responsible for the increased glomerular PTHrP staining in rats with folic acid injury. Our findings indicate that these deposits, which had previously been reported to occur only in the renal tubules, can also occur in the glomerulus. Furthermore, in this segment, some dilated capillaries were observed near the yellowish folic acid deposits in the mesangium, suggesting folic acid deposition in the capillary lumen, which would affect renal microcirculation.

The present study found, to our knowledge for the first time, an up-regulation of ACE mRNA in the kidney of folic acid-treated rats. Interestingly, intense Ang II immunostaining was localized mainly in the nuclei of renal tubular cells in these animals. In this regard, ACE and/or Ang II immunoreactivity previously have been found in the cell nucleus in several tissues, including the kidney [35–37]. In one of these studies, an Ang II-induced nuclear targeting of the Ang II subtype 1 (AT_1) receptor has been demonstrated in rat neurons, which possibly is related to the chronic neuromodulatory effects of Ang II [37]. Whether such a mechanism has a putative role in the folic acid-injured kidney is presently unknown.

We found that the PTH/PTHrP receptor, both its mRNA expression and immunostaining, dramatically decreased in the rat kidney associated with folic acid-induced renal injury. The changes in PTHrP and the PTH/PTHrP receptor as found herein are consistent with, but opposite to, those reported for other growth factors and their receptors, such as for epidermal growth factor and hepatocyte growth factor (HGF), which have a role in the renal regenerative process in this model of acute renal failure [33, 34]. Down-regulation of the PTH/PTHrP receptor, as found in the present study, has been reported to occur in other models of renal injury associated with increased renal PTHrP [4, 15]. In this regard, PTH treatment has been shown to decrease PTH binding and PTH-stimulated cAMP accumulation in renal cells both in vitro and in vivo [38–40]. Moreover, the increase in renal Ang II in folic acid-injected animals also could down-regulate the PTH/PTHrP receptor, as occurs in vascular smooth muscle cells [41]. Indeed, there is evidence suggesting the existence of PTHrP receptors other than the PTH/PTHrP receptor in different tissues and in the kidney [1, 10, 28, 42]. On the other hand, PTHrP and/or the PTH/PTHrP receptor can be internalized into the

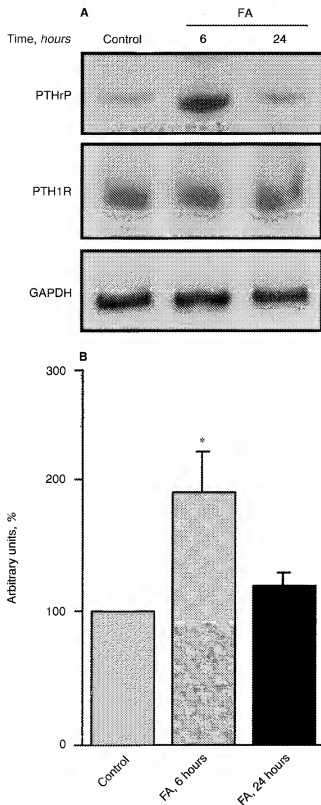


Fig. 10. Effect of folic acid on parathyroid hormone-related protein (PTHrP) and the PTH/PTHrP receptor mRNA in NRK 52E cells. Cells were serum-depleted for 24 hours before the addition of 10 mmol/L folic acid for different time periods. (A) Autoradiograms showing the analysis of PTHrP and the PTH/PTHrP receptor mRNA and GAPDH

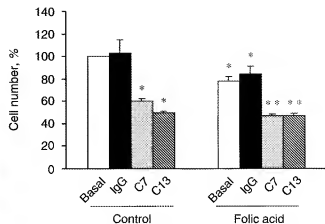


Fig. 11. Effect of folic acid and different anti-PTHrP antibodies on NRK 52E cell number. Subconfluent cells were incubated for 48 hours in serum-depleted medium with or without 10 mmol/L folic acid in the presence or absence of either anti-PTHrP antiserum C7 or C13 or nonimmunogenic rabbit IgG at 5 µg/mL. Then, viable cells were counted in a hemocytometer. Values are mean \pm SEM of results in three to six different cell cultures in triplicate. * $P < 0.01$ compared with antibody-untreated (basal) control value; ** $P < 0.01$ compared with the folic acid-treated basal value.

nucleus in several cell types, including renal cells [43–45]. Clarification of the possible relevance of these mechanisms for the renal effects of PTHrP awaits further studies.

The PTHrP mRNA up-regulation in the kidney of folic acid-injured rats was found to precede the associated increased proliferation in renal tubular cells. In this regard, a recent study has demonstrated that both HGF and its c-met receptor gene expression show a similar response pattern in this rat model of acute renal injury [34]. However, opposite to the increased PTHrP immunostaining found herein, a decrease in HGF protein levels was observed in the kidney of rats with folic acid injury [34]. The results of this study indicate that the renal increase of c-met, which appears to be modulated by various cytokines, appears to be a mechanism participating in the process of renal regeneration in this model [34]. Thus, the possibility exists that PTHrP interacts with this and perhaps other growth factor receptors in renal cells to modulate the renal regenerative response induced by folic acid acute injury.

In the present study, a single dose (3 or 50 µg/kg) of exogenously administered PTHrP (1-36) shortly after folic acid injection significantly increased the number of

mRNA as a constitutive control, using RT-PCR. (B) Changes in PTHrP mRNA levels, corrected to those of GAPDH mRNA, in cells exposed to folic acid. Results are mean \pm SEM of values obtained with total RNA isolated and pooled from three different culture wells in three different cell cultures. * $P < 0.05$ compared with control (100%). FA, folic acid.

PCNA-stained tubular cells at 24 hours, following a peak of PTHrP in plasma. This new finding demonstrates that PTHrP displays mitogenic features for renal tubular cells in vivo. On the other hand, exogenous PTHrP had no effect on the time frame for the recovery of renal function in these rats after folic acid injury. In fact, we found that the decreased renal function did not correlate with tubular cell growth in folic acid-injected rats, treated or not with PTHrP (1-36). It is hypothesized that changes in the renal microvasculature associated with folic acid injury overshadows any putative beneficial effects of PTHrP on renal function following the PTHrP-induced increase in tubular growth in this animal model. Moreover, the changes in Ang II as found herein might trigger an additional deleterious effect on the renal microcirculation in this animal model of acute renal failure. Further studies are needed to test this hypothesis. Our present study also found that PTHrP (1-36) administered in vivo at a dose triggering the renal tubular proliferative effect decreased renal PTHrP mRNA in folic acid-treated rats. This suggests that homologous down-regulation would affect both PTHrP and the PTH/PTHrP receptor expression in renal tissue.

Previous data support the notion that folic acid might induce direct nephrotoxic effects despite its obstructive effects in the renal tubules [18]. Thus, we examined the possible effects of folic acid on the expression of both PTHrP and the PTH/PTHrP receptor and on renal tubular cell growth in renal epithelial cells in vitro. We found that 10 mmol/L folic acid stimulated PTHrP mRNA in rat NRK 52E cells within a time frame similar to that observed in folic acid-treated rats and that it also increased PTHrP levels in the cell-conditioned medium. In contrast, the levels of the PTH/PTHrP receptor mRNA in these cells were unchanged by folic acid over the same time period. Consistent with this finding, post-transcriptional events have been shown to be the main mechanisms for this receptor desensitization in another renal cell line [39]. We found that folic acid decreased the cell number, but this decrease was lower in the presence of anti-PTHrP antibodies in the cell-conditioned medium, suggesting that endogenous PTHrP can counteract in part the antiproliferative effect of folic acid in these cells. Interestingly, and similar to previous findings in other tubular cell preparations [11–13], this autocrine effect of PTHrP also was found to affect normal NRK 52E cell growth.

In summary, the present study found that renal PTHrP was rapidly and transiently increased in rats with folic acid-induced acute renal failure, featuring an early gene response. These findings demonstrate that the exogenous administration of PTHrP (1-36) increases tubular cell proliferation after folic acid injury. Moreover, folic acid, in a similar manner to that in vivo, directly stimulates PTHrP mRNA expression in rat renal epithelial cells in

vitro, which affects the growth of these cells. Our results support the notion that PTHrP plays a role in the regenerative process following acute renal injury induced by folic acid. These findings also lend support to the hypothesis that PTHrP up-regulation is a common event associated with renal injury and repair. In addition, our findings suggest that the renin-angiotensin system could participate in the mechanisms responsible for the altered renal function in this model.

ACKNOWLEDGMENTS

This work was supported in part by grants from Fondo de Investigación Sanitaria of Spain (FIS 96/1167), Programa Sectorial de Promoción General del Conocimiento from the Spanish Ministerio de Educación y Cultura (PM 97/85 and PB98-0700-C02-01 and -02), Consejería de Educación de la Comunidad Autónoma de Madrid (CAM 08.6/0038/2000), and Hospital, S.A. (Barcelona, Spain). We thank A.F. Stewart, M.D., A. García-Ocaña, Ph.D., and F. de Miguel, Ph.D. (Department of Endocrinology, University of Pittsburgh, PA, USA) for the following: providing us with technical know-how and with the PTH/PTHrP receptor plasmid to perform the RNase protection assay in our laboratory and performing the PTHrP assay. We are also grateful to A. Valín, Ph.D., for performing RT-PCR for c-fos gene amplification. S. Santos and R. Largo are fellows of the Spanish Ministerio de Educación y Cultura. Reproduction of Figures 2 and 4 in color was supported by the Spanish Ministry of Science and Technology.

Reprint requests to Pedro Esbri, Ph.D., Laboratorio de Metabolismo Mineral y Óseo, Unidad de Investigación, Fundación Jiménez Díaz, Avda. Reyes Católicos 2, 28004 Madrid, Spain.
E-mail: pesbri@fjd.es

REFERENCES

1. PHILBRICK WM, WYSOLMERSKI JJ, GALBRAITH S, et al: Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol Rev* 76:127–173, 1996
2. AYA K, TANAKA H, ICHINOSE Y, et al: Expression of parathyroid hormone-related peptide messenger ribonucleic acid in developing kidney. *Kidney Int* 55:1696–1703, 1999
3. MASSFELDER T, STEWART AF, ENDLICH K, et al: Parathyroid hormone-related protein detection and interaction with NO and cyclic AMP in the renovascular system. *Kidney Int* 50:1591–1603, 1996
4. SOIFER NE, VAN WHY SK, GANZ MB, et al: Expression of parathyroid hormone-related protein in the rat glomerulus and tubule during recovery from renal ischemia. *J Clin Invest* 92:2850–2857, 1993
5. MASSFELDER T, FIASCHI-TAESH N, STEWART AF, HELWIG J-J: Parathyroid hormone-related peptide: A smooth muscle tone and proliferation regulatory protein. *Curr Opin Nephrol Hypertens* 7:27–32, 1998
6. MARRAS S, SUTCLIFF RL, QIAN J, et al: Targeted overexpression of parathyroid hormone-related protein (PTHrP) to vascular smooth muscle in transgenic mice lowers blood pressure and alters vascular contractility. *Endocrinology* 140:1815–1825, 1999
7. MASSFELDER T, PAREKH N, ENDLICH K, et al: Effect of intrarenally infused parathyroid hormone-related protein on renal blood flow and glomerular filtration rate in the anesthetized rat. *Br J Pharmacol* 118:1995–2000, 1996
8. SAUSSINE C, MASSFELDER T, PARNIN F, et al: Renin stimulating properties of parathyroid hormone-related peptide in the isolated perfused rat kidney. *Kidney Int* 44:764–773, 1993
9. BOSCH RJ, RODRIGUEZ-PUYOL D, BOVER I, RODRIGUEZ-PUYOL M: Parathyroid hormone-related protein: Roles in the glomerulus. *Exp Nephrol* 7:212–216, 1999
10. BOSSER RJ, ROSE-LINARES P, TORRELLAS-CANAMAYOR G, et al: Effects of parathyroid hormone-related protein on human mesangial cells in culture. *Am J Physiol* 277:E990–E995, 1999

11. De MIGUEL F, GARCÍA-CAÑERO R, ESBRIIT P: Copurification of calcium transport-stimulating and DNA synthesis-stimulating agents with parathyroid-like activity isolated from the hypercalcemic strain of the Walker 256 tumor. *Eur J Cancer* 27:1022-1026, 1991
12. GARCÍA-OCAÑA A, De MIGUEL F, PEÑARANDA C, et al: Parathyroid hormone-related protein is an autocrine modulator of rabbit proximal tubule cell growth. *J Bone Miner Res* 10:1875-1884, 1995
13. GARCÍA-OCAÑA A, GALBRAITH SC, VAN WIJCK SK, et al: Expression and role of parathyroid hormone-related protein in human renal proximal tubule cells during recovery from ATP depletion. *J Am Soc Nephrol* 10:238-244, 1999
14. GARCÍA-OCAÑA A, GÓMEZ-CASERO E, PEÑARANDA C, et al: Cyclosporine increases renal parathyroid hormone-related protein expression in vivo in the rat. *Transplantation* 65:860-863, 1998
15. LARGO R, GÓMEZ-GARRÉ D, SANTOS S, et al: Renal expression of parathyroid hormone-related protein (PTHrP) and PTH/PTHrP receptor in a rat model of tubulointerstitial damage. *Kidney Int* 55:82-90, 1999
16. SCHUBERT GE: Folic acid-induced acute renal failure in the rat: Morphological studies. *Kidney Int* 10(Suppl 6):S46-S50, 1976
17. KLINGER EL, EVAN AP, ANDERSON RE: Folic acid-induced renal injury and repair. *Arch Pathol Lab Med* 104:87-93, 1980
18. FINK M, HENRY M, TANGE JD: Experimental folic acid nephropathy. *Pathology* 19:143-149, 1987
19. ESBRIIT P, EGIDO J: The emerging role of parathyroid hormone-related protein as a renal regulating factor. *Nephrol Dial Transplant* 15:1109-1126, 2000
20. HENRY JG, MITNICK M, DANN PR, STEWART AF: Parathyroid hormone-related protein (-1-36) is biologically active when administered subcutaneously to humans. *J Clin Endocrinol Metab* 82:900-906, 1997
21. STEWART F, CAHN RL, BURR DB, et al: Six-month daily administration of parathyroid hormone and parathyroid hormone-related protein peptides to adult ovariectomized rats markedly enhances bone mass and biomechanical properties: A comparison of human parathyroid hormone 1-34, parathyroid hormone-related protein 1-36, and SDZ-parathyroid hormone 893. *J Bone Miner Res* 15:1517-1525, 2000
22. BRADFORD M: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254, 1976
23. ESBRIIT P, BENTÉZ-VERGULAS J, De MIGUEL F, et al: Characterization of parathyroid hormone/parathyroid hormone-related protein receptor and signaling in hypercalcemic Walker 256 tumor cells. *J Endocrinol* 166:11-20, 2000
24. RAMÍREZ C, OLMO A, O'VALLE F, et al: Role of intrarenal endothelin 1, endothelin 3, and angiotensin II expression in chronic cyclosporin A nephrotoxicity in rats. *Exp Nephrol* 8:161-172, 2000
25. WOLF G, HAMANN A, HAN DC, et al: Leptin stimulates proliferation and TGF- β expression in renal glomerular endothelial cells: Potential role in glomerulosclerosis. *Kidney Int* 56:860-872, 1999
26. WHORWOOD CB, STEWART PM: Transcriptional regulation of Na/K-ATPase by corticosteroids, glycyrrhetic acid and second messenger pathways in rat kidney epithelial cells. *J Mol Endocrinol* 93-103, 1995
27. RUIZ-ORTEGA M, BUSTOS C, PLAZA JJ, EGIDO J: Overexpression of extracellular matrix proteins in renal tubulointerstitial cells by platelet-activating-factor stimulation. *Nephrol Dial Transplant* 13:886-892, 1998
28. VALS A, GARCÍA-OCAÑA A, De MIGUEL F, et al: Antiproliferative effect of the C-terminal fragments of parathyroid hormone-related protein, PTHrP (-107-111) and (107-139), on osteoblastic osteosarcoma cells. *J Cell Physiol* 170:209-215, 1997
29. THEIDE M, DAFOTS A, WEIR E, et al: Intrauterine occupancy controls expression of the parathyroid hormone-related peptide gene in pre-term rat myotomium. *Proc Natl Acad Sci USA* 87:6969-6973, 1990
30. PLAWNER LL, PHILLIBRICK WM, BURTIS WJ, et al: Cell type-specific secretion of parathyroid hormone-related protein via the regulated versus the constitutive secretory pathway. *J Biol Chem* 270:14078-14084, 1995
31. YANG T, HASSAN S, HUANG YG, et al: Expression of PTHrP, PTH/PTHrP receptor, and Ca²⁺-sensing receptor mRNAs along the rat nephron. *Am J Physiol* 272:F751-F758, 1997
32. LEE K, BROWN D, UKEA P, et al: Localization of parathyroid hormone/parathyroid hormone-related peptide receptor mRNA in kidney. *Am J Physiol* 270:F186-F191, 1996
33. HUMES HD, LAKE EW, LIU S: Renal tubule cell repair following acute renal injury. *Miner Electrolyte Metab* 21:353-365, 1995
34. LIU Y, TOLBERT EM, LIN L, et al: Up-regulation of hepatocyte growth factor receptor: An amplification and targeting mechanism for hepatocyte growth factor action in acute renal failure. *Kidney Int* 55:442-453, 1999
35. BRUNVAL P, HINGLAIS N, ALHENC-GELAS F, et al: Angiotensin I converting enzyme in human intestine and kidney: Ultrastructural immunohistochemical localization. *Histochemistry* 85:73-80, 1986
36. EDELMAN B, FUXE K, GANTEN D: Subcellular localization of angiotensin II immunoreactivity in the rat cerebellar cortex. *Hypertension* 28:818-824, 1996
37. LU D, YANG H, SHAW G, RAIZADA MK: Angiotensin II-induced targeting of the angiotensin type 1 (AT1) receptor in brain neurons. *Endocrinology* 139:365-375, 1998
38. ABOU-SAMRA A-B, GOLDSMITH PK, XIE LY, et al: Down-regulation of parathyroid hormone (PTH)/PTH-related peptide receptor immunoreactivity and PTH binding in opossum kidney cells by PTH and dexamethasone. *Endocrinology* 135:2588-2594, 1994
39. UREÑA P, IJUA-KLEIN A, KONG X-F, et al: Regulation of parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acid by glucocorticoids and PTH in ROS 17/2.8 and OK cells. *Endocrinology* 134:451-456, 1994
40. MAHONEY CA, NISSENSEN RA: Canine renal receptors for parathyroid hormone: Down-regulation in vivo by exogenous parathyroid hormone. *J Clin Invest* 72:411-421, 1983
41. OKANO K, WU S, HUANG X, et al: Parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor and its messenger ribonucleic acid in rat aortic vascular smooth muscle cells and UMR osteoblast-like cells: Cell-specific regulation by angiotensin-II and PTHrP. *Endocrinology* 135:1093-1099, 1994
42. JOBERT A-S, FERNANDES I, TURNER G, et al: Expression of alternatively spliced isoforms of the parathyroid hormone (PTH)/PTH-related peptide receptor messenger RNA in human kidney and bone cells. *Mol Endocrinol* 10:1066-1076, 1996
43. HENDERSON JE, AMIZUKA N, WARSHAWSKY H, et al: Nuclear localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol Cell Biol* 15:4064-4075, 1995
44. MASSFELDER T, DANN P, WU TL, et al: Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: A critical role for nuclear targeting. *Proc Natl Acad Sci USA* 94:13630-13635, 1997
45. WATSON PH, FRANKEL LJ, HENDY GN, et al: Nuclear localization of the type I PTHrP/PTHrP receptor in rat tissues. *J Bone Miner Res* 15:1033-1044, 2000

Transplantation®

BRIEF COMMUNICATION

CYCLOSPORINE INCREASES RENAL PARATHYROID HORMONE-RELATED PROTEIN EXPRESSION IN VIVO IN THE RAT¹

ADOLFO GARCIA-OCANA, ELENA GOMEZ-CASERO, CARLOS PEÑARANDA, JOSE LUIS SARASA, AND PEDRO ESBRI²

Research Unit and Department of Pathology, Fundación Jiménez Díaz, 28040 Madrid, Spain

Background. Clinical use of cyclosporine (CsA) is limited by its known nephrotoxicity. Parathyroid hormone (PTH)-related protein (PTHrP) increases after acute renal ischemia and stimulates proliferation of renal cells in culture. Herein, we have examined whether the renal expression of PTHrP and its PTH/PTHrP receptor is affected by chronic CsA nephrotoxicity.

Methods. Rats were randomly assigned to receive daily intramuscular injections of either CsA (25 mg/kg) or the same volume of the vehicle olive oil (control) for 3 weeks. At this time interval, under ether anesthesia, rat blood and kidneys were obtained for analytical determinations, and total RNA isolation or immunohistochemistry, respectively.

Results. Serum urea was 11 ± 2 and 6 ± 1 mmol/L ($P < 0.01$) in CsA-treated and control rats, respectively. We found that PTH/PTHrP receptor mRNA was unchanged, but PTHrP mRNA, and also transforming growth factor- β mRNA expression as positive control, was about twofold increased in the kidney of CsA-treated rats. This was accompanied by increased PTHrP immunostaining in renal cortical tubules, associated with tubule vacuolization.

Conclusion. This study demonstrates an up-regulation of PTHrP, associated with chronic CsA-induced nephrotoxicity. Our findings support a role for PTHrP in the CsA-injured kidney.

The fungal peptide cyclosporine (CsA*) has proven to be a powerful tool in the prevention of allograft rejection. However, clinical use of CsA is limited by its unpredictable nephrotoxicity, the pathophysiology of which is unclear. Several studies indicate that CsA nephrotoxicity is associated with preglomerular vasoconstriction, mediated at least in part by the CsA-induced increase of vasoactive growth factors (1, 2). In addition, the antiproliferative effects of CsA on renal proximal tubule cells in culture could contribute to the mechanism of tubular injury in CsA-treated subjects (3).

Parathyroid hormone (PTH)-related protein (PTHrP) was originally purified from tumors associated with hypercalcemia and is now known to be the main factor responsible for the latter paraneoplastic syndrome. PTHrP is also present in a variety of normal tissues, including the kidney, where it appears to affect cell growth and/or cell differentiation in an autocrine fashion (4). In the kidney, the physiological role of PTHrP is unclear, but it has recently been shown to stimulate growth in rat mesangial cells and rabbit proximal tubule cells (5, 6). Interestingly, the mitogenic effect of PTHrP in the latter cells occurs in subconfluent but not in confluent cell cultures (6). Moreover, PTHrP increases in the rat renal tubule during the recovery phase after acute renal ischemia (5). Thus, PTHrP could be produced as a general response to renal tubular injury.

In this study, we have investigated the expression of PTHrP and its PTH/PTHrP receptor in the kidney of CsA-treated rats. We have also examined the renal expression of transforming growth factor (TGF)- β , which is known to increase in rats with CsA-induced nephrotoxicity (7).

The experiments were carried out on male Wistar rats (250 g), which were divided into two groups. Six rats received daily intramuscular injections of CsA (Sandimmune, Sandoz, Basel, Switzerland) (25 mg/kg body weight), and another six animals were injected with an equivalent volume of the olive oil vehicle, for up to 3 weeks. One day before and at the end of the experimental period of study, the rats were put into metabolic cages and were deprived of food for 18 to 22 hr but allowed free access to tap water. Then, the animals were weighed, blood was taken by cardiac puncture under ether anesthesia, and excreted urine was collected. Serum and urine creatinine and serum urea were determined by autoanalyzer (Dimension AR, DuPont, Wilmington, DE). CsA in whole blood was measured by immunofluorescence (Abbott, Germany).

Statistical analysis was performed using the unpaired Student's *t* test, and $P < 0.05$ was considered significant.

Over the 3-week treatment period, the gain in body weight of CsA-treated rats was on average about 20% of that of the control group of animals. Creatinine and urea in serum were significantly higher (71 ± 9 μ mol/L and 11 ± 2 mmol/L, respectively) and creatinine clearance was lower (0.24 ± 0.04 ml/min/100 g body weight) in the CsA-treated group compared with the control group (58 ± 9 μ mol/L $P < 0.05$, 6 ± 1 mmol/L

¹ This study was supported in part by a grant from the Spanish Fondo de Investigación Sanitaria (FIS 96/1167).

² Address correspondence to: P. Esbri, Ph.D., Research Unit, Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain.

* Abbreviations: CsA, cyclosporine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RT-PCR, reverse transcription polymerase chain reaction; TGF, transforming growth factor.

March 27, 1998

BRIEF COMMUNICATION

861

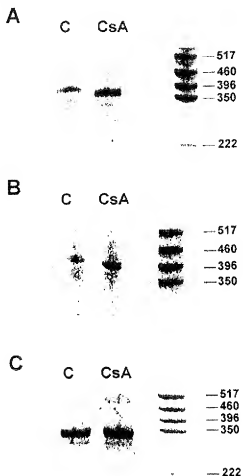


FIGURE 1. PTHrP (A), TGF- β_1 (B), and PTHrP receptor (C) mRNA expression in the kidney from CsA-treated and control (c) rats. RT-PCR was carried out as described in the text. PCR products were electrophoresed on 5% polyacrylamide gels. The first column on the right are DNA markers. The figure is representative of results obtained with six animals per group.

[$P < 0.01$], and 0.31 ± 0.09 ml/min/100 g body weight [$P < 0.05$], respectively). Whole blood CsA was 2250 ± 806 μ g/L at the end of treatment with CsA, whereas it was undetectable in the blood of rats in the control group. Systolic blood pressure was not tested in our experimental animals. However, previous studies did not find significant changes in this parameter after more prolonged treatment with the same CsA dose as used in the present study (1, 7).

To determine whether PTHrP and the PTHrP receptor are overexpressed in the kidney of CsA-treated rats, we used semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and/or immunohistochemistry (8).

At the end of the experimental treatment, under ether anesthesia, the rat kidneys were perfused with phosphate-buffered saline (pH 7.4). They were then removed and either frozen immediately in liquid nitrogen or fixed overnight in 10% formalin and embedded in paraffin.

Whole rat kidney tissue was homogenized with a glass-Teflon homogenizer in 4 M guanidine isothiocyanate and 0.1

M β -mercaptoethanol, and total RNA was isolated. RT was carried out with Moloney murine leukemia virus reverse transcriptase (Pharmacia, Uppsala, Sweden) at 37°C for 1 hr. Resulting cDNA was then subjected to submaximal PCR amplification with *Taq* DNA polymerase (BIOTAQ, Bioline, London, UK) in a reaction mixture (with 1 μ Ci of [α^{32} P]dCTP) containing sequence-specific primers: 5'-TG-CAGCGGAGACTGGTTCAG-3' and 5'-CCTCGTCGTCTGACCAAA-3' (PTHrP); 5'-AGGTGGTTCCAGGGCACAA-3' and 5'-CAACTCTTCCCTCTGTGAGGC-3' (PTHrP receptor); 5'-TTCCTGCTCTCATGCCCAC-3' and 5'-AGGACGCGCAGCATCATGTG-3' (TGF- β_1 as positive control); and 5'-TCCTGCCACCACTGCTTA-3' and 5'-ACCACCTCTGTGTGTAGCC-3' (glyceraldehyde 3-phosphate dehydrogenase [GAPDH] as a constitutive control). The PCR products were separated on 5% polyacrylamide gels and quantified by densitometric scanning after exposure to radi-sensitive film. The PCR amplification products were analyzed for authenticity by restriction enzyme digestion and/or sequencing.

As shown in Figure 1, expression of both PTHrP mRNA and TGF- β_1 mRNA were induced in the kidney after 3 weeks of treatment with CsA. The amount of PTHrP mRNA and that of TGF- β_1 mRNA, expressed as a ratio over that of GAPDH, which was similar in CsA-treated and -untreated rats (not shown), were about twofold higher than those of control animals (Fig. 2).

Our observation of an increased TGF- β_1 gene expression in the whole rat kidney after CsA treatment is consistent with recent findings in rodents treated with a CsA dose similar to that used in the present study (7, 9). These studies support the current concept that TGF- β_1 , a multifunctional cytokine with cell growth inhibitory and fibrogenic features (10), is involved in the immunosuppressive and nephrotoxic effects of CsA.

We also assessed whether the increased renal expression of PTHrP mRNA was accompanied by an increase of the protein.

Paraffin-embedded tissue sections (5 μ m) were incubated with 5 μ g/ml affinity purified anti-PTHrP antibody Ab-2 (Oncogene Science, Uniondale, NY), recognizing the sequence (34–53) of human and rat PTHrP, and subsequently

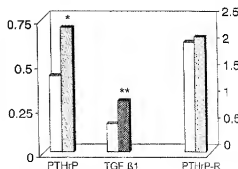


FIGURE 2. The mean PTHrP, TGF- β_1 , and PTHrP receptor (PTHrP-R) to GAPDH ratio for the CsA-treated (shadow bars) and control (open bars) groups studied. Densitometric values in arbitrary units were used for ratio calculation. The left and right axes correspond to the latter ratio values for PTHrP and TGF- β_1 , and for the PTHrP-R, respectively. * $P < 0.01$; ** $P < 0.05$, compared with the corresponding control.

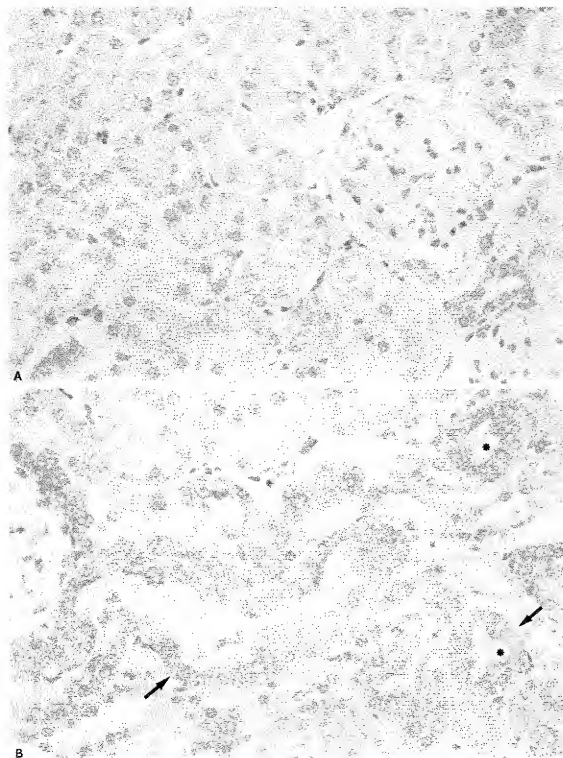


FIGURE 3. Immunohistochemical staining for PTHrP in kidney tissue sections from control (A) and CsA-treated (B) rats, with anti-PTHrP (34-53) antibody. Representative areas of intense staining associated with tubule vacuolization and brush border are indicated by arrows and asterisks, respectively (original magnification: $\times 430$).

immunostained by the avidin-biotin-peroxidase complex method (11). Sections were routinely counterstained with hematoxylin and eosin.

Addition of anti-PTHrP antibody to kidney sections from

vehicle-treated rats revealed weak and diffuse cytoplasmic tubular staining (Fig. 3A). On light microscopic examination, kidneys from CsA-treated rats showed tubule dilatation with irregular epithelium and vacuolization (Fig. 3B).

These tubules, and their brush border, were found to stain intensely for PTHrP (Fig. 3B). Positive PTHrP signal was also observed in the vascular system, when present in the tissue section, and it was similar in both CsA-treated and control animals. Omission of the primary antibody produced no staining.

These changes in staining and/or gene expression are not likely to be explained by a lower food intake in CsA-treated rats, since they were consistently observed in all of these animals independently of their weight gain, which was variable. Moreover, an increase in renal TGF- β_1 expression has been shown to occur in rats and mice treated with a CsA dose similar to that used in the present study, using an experimental design including or not pair-fed controls (7, 9).

The mechanisms responsible for the *in vivo* up-regulation of renal PTHrP by CsA are unknown. The vasoconstricting peptide endothelin has been shown to be stimulated by CsA in proximal tubule cells (12). Endothelin and TGF- β induce PTHrP mRNA in rat aortic smooth muscle cells and human myometrial cells, respectively (4). Therefore, these mechanisms might contribute to the *in vivo* stimulation of PTHrP in the renal tissue by CsA.

A previous study has shown an overexpression of PTHrP in proximal tubule cells during the repair process after acute renal ischemia (5). In light of the growth effects of PTHrP in various kidney cell types (5, 6), the latter results and those of this study of a model of chronic nephropathy, taken together, support the concept that PTHrP could play a regulatory role during renal regeneration.

In our study, we could not detect any effect of CsA on PTHrP immunostaining in renal arterioles, when present in the kidney sections. However, we cannot rule out the possibility that vascular production of PTHrP is increased in this situation, due to the relatively low sensitivity of the immunohistochemical technique. Considering the vasorelaxant effect of PTHrP (13), its increase in the kidney might modulate the vessel contractility associated with CsA-induced nephrotoxicity.

PTHrP/PTHrP receptor mRNA in the kidney from CsA-treated rats was unchanged compared with that of the control group (Figs. 1 and 2). PTHrP/PTHrP receptor down-regulation associated with an increased PTHrP has been shown to occur in other systems, including the rat kidney during recovery after acute renal ischemia (5). Recently, PTHrP/PTHrP receptor mRNA in the kidney of uremic rats after % nephrectomy was found to decrease compared with that of normal animals (14). In the latter study though, plasma creatinine was fourfold increased in the nephrectomized rats, which indicates a more severely impaired renal function than that of the CsA-treated rats in the present study. In fact, the level of PTHrP/PTHrP receptor mRNA appeared to correlate inversely with the degree of renal failure in the former animals (14). Nevertheless, our data do not rule out a putative PTHrP/PTHrP receptor down-regulation induced by the observed increase of PTHrP, which could have been overshadowed by a possible stimulatory effect of CsA on this receptor mRNA expression.

In summary, the present study demonstrates, for the first time, to our knowledge, that PTHrP is up-regulated at the mRNA and protein levels in the rat kidney *in vivo* by chronic treatment with CsA. Our findings suggest a multiple function for PTHrP in the damaged kidney.

Acknowledgements. The authors thank Dr. R. Gazapo (Department of Clinical Biochemistry) for creatinine, urea, and CsA determinations. They also thank T. Carrizosa (Department of Pathology) for technical assistance. A.G.-O. and C.P. were recipients of a fellowship from Fondo de Investigación Sanitaria (FIS) and Fundación Conchita Rabago, respectively.

REFERENCES

- Shehata M, El Nahas AM, Barkworth E, et al. Increased platelet-derived growth factor in the kidneys of cyclosporin-treated rats. *Kidney Int* 1994; 46: 726.
- Kon V, Suguru M, Inagami T, et al. Role of endothelin in cyclosporin-induced glomerular dysfunction. *Kidney Int* 1991; 37: 1487.
- Ryffel B, Foxwell BM, Goe A, et al. Cyclosporine—relationship of side effects to mode of action. *Transplantation* 1988; 46 (suppl): 908.
- Philbrick WM, Wysocki JJ, Galbraith S, et al. Defining the roles of parathyroid hormone-related protein in normal physiology. *Endocr Rev* 1996; 17: 127.
- Soifer NE, Van Why SK, Ganz MB, et al. Expression of parathyroid hormone-related protein in the rat glomerulus and tubule during recovery from renal ischemia. *J Clin Invest* 1993; 92: 2850.
- García-Ocaña A, de Miguel F, Peñaranda C, et al. Parathyroid hormone-related protein is an autocrine modulator of rabbit proximal tubule cell growth. *J Bone Miner Res* 1995; 10: 1875.
- Shihab FS, Andoh TF, Tanner AM, et al. Role of transforming growth factor- β_1 in experimental chronic cyclosporine nephropathy. *Kidney Int* 1996; 49: 1141.
- Peñaranda C, García-Ocaña A, Sarasa JL, Esbrit P. Hypertrophy of rabbit proximal tubule cells is associated with overexpression of TGF- β . *Life Sci* 1996; 59: 1773.
- Khanna A, Kapur S, Sharma V, et al. *In vivo* hyperexpression of transforming growth factor- β_1 in mice: stimulation by cyclosporine. *Transplantation* 1997; 63: 1037.
- Moses HL, Yang EY, Pietenpol JA. TGF- β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 1990; 63: 245.
- Albar JP, de Miguel F, Esbrit P, et al. Immunohistochemical detection of parathyroid hormone-related protein in a rare variant of hepatic neoplasm (sclerosing hepatic carcinoma). *Hum Pathol* 1996; 27: 728.
- Ong ACM, Jowett TP, Scobie JE, et al. Effect of cyclosporin A on endothelial synthesis by cultured human renal cortical epithelial cells. *Nephrol Dial Transplant* 1993; 8: 748.
- Massfelder T, Stewart AF, Endlich K, et al. Parathyroid hormone-related protein detection and interaction with NO and cyclic AMP in the renovascular system. *Kidney Int* 1996; 50: 1591.
- Ureña P, Kubrasly M, Mannstadt M, et al. The renal PTHrP/PTHrP receptor is down-regulated in rats with chronic renal failure. *Kidney Int* 1994; 45: 606.

Received 24 June 1997.

Accepted 17 December 1997.

Animal Model

Humoral Hypercalcemia of Malignancy

Severe Combined Immunodeficient/Beige Mouse Model of Adult T-Cell Lymphoma Independent of Human T-Cell Lymphotropic Virus Type-1 Tax Expression

Virgile Richard,* Michael D. Lairmore,* Patrick L. Green,* Gerold Feuer,[†] Robert S. Erbe,* Björn Albrecht,* Celine D'Souza,* Evan T. Keller,[‡] Jinlu Dai,[§] and Thomas J. Rosol*

From the Department of Veterinary Biosciences,* College of Veterinary Medicine, The Ohio State University, Columbus, Ohio; the Department of Microbiology and Immunology,[‡] State University of New York Upstate Medical University, Syracuse, New York; and the Unit for Laboratory Animal Medicine and Department of Pathology,[§] School of Medicine and Institute for Gerontology, University of Michigan, Ann Arbor, Michigan

The majority of patients with adult T-cell leukemia/lymphoma (ATL) resulting from human T-cell lymphotropic virus type-1 (HTLV-1) infection develop humoral hypercalcemia of malignancy (HHM). We used an animal model using severe combined immunodeficient (SCID)/beige mice to study the pathogenesis of HHM. SCID/beige mice were inoculated intraperitoneally with a human ATL line (RV-ATL) and were euthanized 20 to 32 days after inoculation. SCID/beige mice with engrafted RV-ATL cells developed lymphoma in the mesentery, liver, thymus, lungs, and spleen. The lymphomas stained positively for human CD45RO surface receptor and normal mouse lymphocytes stained negatively confirming the human origin of the tumors. The ATL cells were immunohistochemically positive for parathyroid hormone-related protein (PTHrP). In addition, PTHrP mRNA was highly expressed in lymphomas when compared to MT-2 cells (HTLV-1-positive cell line). Mice with lymphoma developed severe hypercalcemia. Plasma PTHrP concentrations were markedly increased in mice with hypercalcemia, and correlated with the increase in plasma calcium concentrations. Bone densitometry and histomorphometry in lymphoma-bearing mice revealed significant bone loss because of a marked increase in osteoclastic bone resorption. RV-ATL cells

contained 1.5 HTLV-1 proviral copies of the *tax* gene as determined by quantitative real-time polymerase chain reaction (PCR). However, *tax* expression was not detected by Western blot or reverse transcriptase (RT)-PCR in RV-ATL cells, which suggests that factors other than Tax are modulators of PTHrP gene expression. The SCID/beige mouse model mimics HHM as it occurs in ATL patients, and will be useful to investigate the regulation of PTHrP expression by ATL cells *in vivo*. (*Am J Pathol* 2001, 158:2219-2228)

Humoral hypercalcemia of malignancy (HHM) is an important paraneoplastic syndrome occurring in humans with a wide variety of cancers.¹ Parathyroid hormone-related protein (PTHrP) was originally isolated from specific tumors as the primary cause of HHM² and is overexpressed by many types of neoplasms.³ PTHrP is a polypeptide hormone discovered in 1987 and is structurally similar to parathyroid hormone (PTH).⁴⁻⁶ Amino-terminal peptides of PTHrP have been shown to exert PTH-like actions in bone and kidney by binding to a common receptor for PTH/PTHrP (PTH-1 receptor) and resulting in hypercalcemia.⁷⁻⁹ The primary mechanism for bone loss and hypercalcemia in patients with lymphoma is increased osteoclastic bone resorption induced by humoral mediators produced by neoplastic cells.¹⁰ It has been demonstrated that expression of PTHrP mRNA in both humans and animals correlates well with the occurrence of hypercalcemia and hypercalcemia in tumor-

Supported by National Institutes of Health, United States Public Health Service grants CA-77911 and RR-00168 (to T. J. R.), CA-77556 (to P. L. G.), RR-14324 and AI-01474 (to M. D. L.), and CA-77567 (to G. F.); and the C. Glenn Barber Fellowship (to V. R.) from the College of Veterinary Medicine, The Ohio State University.

Accepted for publication February 16, 2001.

Address reprint requests to Dr. Thomas J. Rosol, Department of Veterinary Medicine, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Rd., Columbus, OH, 43212. E-mail: rosol.1@osu.edu.

bearing animals is corrected by a neutralizing antibody to PTHrP.^{11,12}

Adult T-cell leukemia/lymphoma (ATL) is an aggressive and often fatal malignancy of helper/inducer T lymphocytes (CD4) caused by infection with a complex retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1).^{13,14} Hypercalcemia is frequently observed in ATL patients and represents a life-threatening complication of this disease.¹⁵ The virus contains, in addition to *gag*, *pol*, and *env* genes, a regulatory gene region (pX) that encodes several proteins from four open reading frames, including Tax.^{16,17} Tax is a 40-kD nuclear-localizing phosphoprotein that increases viral transcription from the HTLV-1 LTR, as well as many cellular genes including interleukin (IL)-2, IL-2 receptor (IL-2R) α chain,¹⁸ and PTHrP.¹⁹ It also has been reported that Tax transactivates the PTHrP gene promoter *in vitro*.²⁰ In contrast, little is known about regulation of the PTHrP gene in HTLV-1 lymphoma cells *in vivo*. Moreover, very low levels of tax mRNA are present in ATL cells *in vivo*.^{21,22} Thus, factors other than Tax are likely responsible for transcription of the PTHrP gene in ATL cells *in vivo*. ATL cells from patients can secrete other humoral factors such as IL-1 β ,²³ transforming growth factor- β (TGF- β),²⁴ and tumor necrosis factor- β (TNF- β).²⁵ These cytokines have been shown to induce bone resorption in humans.¹ Moreover, IL-2 increases PTHrP production and secretion in HTLV-1-infected T cells.^{26,27} In addition, PTHrP and IL-6 act synergistically in the development of hypercalcemia in patients with hematological malignancies.²⁸ Other cytokines, such as TGF- β , TNF- α , and IL-1, up-regulate PTHrP gene expression in a variety of non-lymphoid cell lines and tissues.^{29,30} Nevertheless, little is known about their action on PTHrP expression in HTLV-1-infected lymphocytes.

We have previously reported development of lymphoma in severe combined immunodeficient (SCID) mice inoculated with peripheral blood lymphocytes from ATL patients.³¹ However, SCID/beige (bg) mice were chosen in the present study because they previously have been shown to have a greater efficiency for xenografting neoplastic human lymphoid tissue in comparison with SCID mice. SCID/bg mice lack natural killer (NK) cell activity and have macrophage defects in addition to lack of functional B and T lymphocytes.

There is little new information on the pathogenesis of HHM in humans because of the lack of relevant *in vivo* models. We have demonstrated that ATL cells develop lymphoma in SCID/beige mice, and animals consequently develop HHM as observed in human patients. This new model will permit mechanistic studies on the interrelationships between cytokines and PTHrP in the pathogenesis of HHM.

Materials and Methods

Animals and ATL Cell Inoculation

Immunodeficient SCID/bg (C.B-17/1crCr-scld-bgBR) mice (Charles River Laboratories, Inc., Wilmington, MA) were maintained under specific pathogen-free conditions

in the animal facility of the College of Veterinary Medicine at The Ohio State University (Columbus, Ohio). Male mice (5 weeks-of-age) were used as recipients, anesthetized with xylazine-ketamine, and injected intraperitoneally with 4×10^7 RV-ATL cells suspended in RPMI 1640 medium. Controls were inoculated with medium alone. The source of the RV-ATL cell line was previously described.³²

Histology and Immunohistochemistry

Animals from the early control group (animals C1 to C7) and early ATL group (animals L1 to L7, L26, and L28) were sacrificed at day 20. Animals from late control group (animals C8 to C14) and late ATL group (animals L8 to L25, L27, L29, L30, and L31) were sacrificed between days 29 and 32 after inoculation. A complete necropsy was performed on each animal. Heart, lungs, thymus, kidneys, liver, stomach, small intestine, colon, mesentery, liver, spleen, vertebrae, and pancreas were fixed immediately after removal from the sacrificed animals in 10% neutral-buffered formalin, embedded in paraffin, cut into 5- μ m-thick sections, and stained with hematoxylin and eosin. Immunohistochemistry was performed on paraffin sections with the following primary antibodies: polyclonal rabbit anti-PTHrP (PTHrP amino acids 34 to 53) (1:100; Ab-2, Oncogene Research Products, Cambridge, MA), and monoclonal mouse anti-CD45RO (1:100; DAKO, Carpinteria, CA). Visualization was achieved using an avidin-biotin complex (Pierce, Rockford, IL), color development with diaminobenzidine (Research Genetics, Huntsville, AL), and hematoxylin counterstain. Negative control slides were stained with omission of the primary antibody. Normal human skin sections served as positive controls.

Measurement of Plasma Calcium and PTHrP Concentrations

Calcium and PTHrP concentrations were measured in early control (C2 to C7) and late control (C8 to C14) animals, as well as, in lymphoma-bearing animals from early ATL (L1, L3 to L5, L7, L26, and L28), and late ATL (L14, L15, L17, L20, L21, L23, L25, L27, and L29 to L31) groups. Blood was obtained from the femoral artery at necropsy. Ionized calcium concentrations were measured with a Nova 8 electrolyte/chemistry analyzer (Nova Biomedical, Waltham, MA). Total calcium concentrations were measured by colorimetric assay (Sigma Chemical Co., St. Louis, MO). Plasma PTHrP concentrations were determined by a two-site immunoradiometric assay (DiaSorin, Stillwater, MN) specific for the PTHrP N-terminal region (amino acids 1 to 40) and mid-region (amino acids 57 to 80).

Bone Histomorphometry and Mineral Densitometry

Lumbar vertebrae were collected and fixed in 10% neutral-buffered formalin for 24 hours at 4°C, decalcified in

10% ethylenediaminetetraacetic acid (pH 7.4) at 4°C, dehydrated in graded series of ethanol for 5 days at 4°C, infiltrated in two changes of glycol methacrylate (Poly-sciences Inc., Warrington, PA) for 10 days at 4°C, and embedded in glycol methacrylate at 4°C. Sections were cut at 5 μ m, histochemically stained for tartrate-resistant acid phosphatase (Sigma Co.), and counterstained with hematoxylin. Histomorphometry of bones was completed in mice with lymphoma from early ATL (L1, L3 to L5, L7, and L26) and late ATL (L8, L11, L12, L13, L14, L15, L17, L19, L20, L23, L27, and L29 to L31) groups, as well as, age-matched control mice (C1 to C3, C5, C7, C8, and C10 to C14) with Bioquant Nova Image Analysis Software (R&M Biometrics Inc., Nashville, TN). Measurement of osteoclastic bone resorption was completed on trabecular bone and osteoclasts were identified as cells lining trabecular bone that stained intensely fuchsia for tartrate-resistant acid phosphatase. Measurements included total bone area, trabecular bone area and perimeter, osteoclast number/mm trabecular bone, and percent osteoclast perimeter.

Bone mineral density (BMD) was measured in early control (C1 to C7), late control (C8 to C14), early ATL (L1, L3 to L5, and L7), and late ATL (L8 to L15, L17, L19 to L21, L23, and L25) groups using dual-energy X-ray absorptiometry on an Eclipse peripheral DEXA Scanner (Norland, Ft. Atkinson, WI) using research software. To measure femoral BMD, the right femur was excised from soft tissue and placed on the scanner in lateral position. The femur was scanned at 5 mm/s with a resolution of 0.1 mm \times 0.1 mm. Total femoral BMD was determined in a window that encompassed the entire femur. Distal metaphyseal BMD was measured in a window that originated 0.25-cm proximal from the distal epiphysis and extended proximally for 0.25 cm.

Northern Blot Analysis of PTHrP Expression

Mesenteric lymphomas were snap-frozen in liquid nitrogen. Total RNA was isolated using Trizol (Life Technologies, Inc., Grand Island, NY). Total RNA (40 μ g) was separated on a 1.2% agarose-formaldehyde gel, transferred to Duralon UV membranes (Stratagene, La Jolla, CA), and crosslinked using a UV Stratilinker 1800 (Stratagene). The membranes were hybridized for 3 hours at 68°C with a ³²P-dATP-labeled probe for human PTHrP (clone 661).³³ The membranes were exposed to Kodak X-OMAT AR film (Kodak, Rochester, NY) for 24 hours at -80°C for autoradiography.

HTLV-1 Provirus Copy Number in RV-ATL Cells

Genomic DNA from cells was obtained using the Qiaamp Blood Kit (Qiagen, Valencia, CA). Genomic DNA (50 ng) was amplified using a Roche Molecular Biochemicals Light Cycler in triplicate samples (Roche Molecular Biochemicals, Indianapolis, IN). Amplification was performed in the presence of 4 mmol/L of MgCl₂ using primer pairs specific for the *tax* (670/671) and *gag* (SG166/SG296) genes³⁴ of HTLV-1. Cycling conditions

were as follows: 2 minutes at 94°C for denaturation, and 60 cycles of 94°C for 1 second, 55°C for 1 second, and 72°C for 10 seconds. After the last amplification cycle, a melting curve analysis was performed to determine the specificity of the PCR reaction. Proviral copy number was determined using a standard curve obtained by amplification of a serially diluted plasmid (ACH) representing an infectious molecular clone of HTLV-1³⁵ or StyΔ28 that contains a 588-bp fragment of the *gag* gene.³⁶

Western Blot Analysis of HTLV-1 Gene Products

Approximately 1×10^7 RV-ATL cells were collected by peritoneal lavage from lymphoma-bearing SCID/bg mice. Other HTLV-1 infected and noninfected cells (MT-2, HT1-RV, Jurkat, and SLB-1 cell lines) were maintained in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were lysed (0.15 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 10 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L NaF, 0.5% deoxycholate, 50 mmol/L Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 10% glycerol, 1% Nonidet P-40) on ice for 10 minutes. Cell lysates were centrifuged at 17,000 \times g for 10 minutes at 4°C. Lysate protein concentrations were determined using the BioRad (Hercules, CA) microassay. Equivalent amounts of protein (40 μ g) were mixed with 2 \times sample buffer (0.08 mol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.1 mol/L dithiothreitol, 10% glycerol, 0.1% bromophenol blue). The samples were boiled for 3 minutes and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane (BA-79; Schleicher & Schuell, Keene, NH) for 2 hours at 40 V. The membrane was washed three times with TBS-T (9% NaCl, 0.1 mol/L Tris base, 0.1% Tween 20, pH 7.2) and blocked in a 5% powdered milk/TBS-T solution overnight at 4°C. The membrane was washed three times and incubated with rabbit polyclonal anti-Tax (1:500; AIDS Reagent Program, Rockville, MD), mouse monoclonal anti-p19 (1:100; Zep-toMatrix Corporation, Buffalo, NY), mouse monoclonal anti-p24 (1:100; Genzyme Corporation, Cambridge, MA), and mouse monoclonal anti-gp46 (1:500)³⁷ overnight at 4°C. The membrane was washed three times with TBS-T, and incubated with rabbit horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia, Piscataway, NJ) for 1 hour. After a final wash with TBS-T, the HTLV-1 proteins were detected by enhanced chemiluminescence detection reagents (Amersham Pharmacia) and developed on BioMax MR Film (Kodak, Rochester, NY).

RT-PCR for HTLV-1 Tax/Rex RNA

Total cellular RNA was extracted from 729, HT1-RV, SLB-1, MT-2 cells, and from xenografted RV-ATL cells using the Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) procedure.³⁸ All RNA was digested three times with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN), precipitated, and quantified by absorbance at 260 and 280 nm. Approximately 600 ng of RNA

2222 Richard et al
AJP June 2001, Vol. 158, No. 6

Table 1. Gross Pathology and Histopathology in SCID/bg Mice Xenografted with Human ATL Cells

Group	Gross pathology				Histopathology						
	Mesenteric lymphoma	Splenomegaly	Thymus enlargement	Pericardial mineralization	Lymphoma						Pericardial mineralization
					Mesentery	Spleen	Liver	Thymus	Lungs	Bone	
Early Lymphoma (day 20)	7/9	3/9	0/9	0/9	7/9	3/9	0/9	3/9	0/9	0/9	0/9
Late lymphoma (day 29 to 32)	19/22	8/22	2/22	9/22	19/22	5/22	17/22	17/22	2/22	5/22	13/22
Total	26/31	11/31	2/31	9/31	26/31	8/31	17/31	20/31	2/31	5/31	13/31

was amplified by a coupled primer extension-30 cycle PCR reaction containing HTLV-1-specific oligonucleotide primer pairs. The coupled primer extension-PCR reaction (50 μ l) contained RNA, 0.25 mmol/L deoxynucleoside triphosphates, 50 mmol/L KCL, 10 mmol/L Tris (pH 8.0), 1.5 mmol/L MgCl₂, 0.01% gelatin, 100 ng 3' (antisense) oligonucleotide, 50 ng 5' (sense) oligonucleotide end-labeled with T4 DNA kinase to a specific activity of $\sim 2 \times 10^6$ cpm/ μ g, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) in the presence (+) or absence (-) of 5 U of murine leukemia virus reverse transcriptase (Amersham Pharmacia). The reaction was performed in a Perkin Elmer thermal cycle 9600: 65°C for 10 minutes, 50°C for 8 minutes, and 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes. PCR-amplified products were separated on a 6% polyacrylamide gel and visualized by autoradiography. The sequence of the HTLV-1-specific oligonucleotides were; LA79 HTLV-1-5' GTC CAA ACC CTG GGA AGT GG 3'; LA78 HTLV-1-5' CAA GTG GAT CCC GTG GAG AT 3'. The primer pair was designed to amplify spliced tax/rex-specific RNA (117 bp). A second primer pair (670/671) was used, as previously described³⁹, to amplify a region of the tax/rex gene, but it also detects all species (spliced and nonspliced) of viral RNA (159 bp).

Statistical Analysis

Numerical data were expressed as means \pm SD. Statistical differences between means for the different groups were evaluated with Instat 3.01 (GraphPAD software) using one-way analysis of variance, Bonferroni multiple comparisons test, and paired t-test with the level of significance at $P < 0.05$. Correlation coefficients were determined by linear regression.

Results

Xenografted ATL Cells Grow in SCID/bg Mice

ATL cells inoculated intraperitoneally into 5-week-old SCID/bg mice produced diffuse lymphoma in the mesentery of 84% (26 of 31) of the mice between 20 to 32 days after inoculation (Table 1). At necropsy, mice in the early ATL group (day 20) had mild to moderate ascites (3 to 4 ml) containing a suspension of ATL cells recovered by abdominal lavage. Small tumors were present at the root of the mesentery in 78% (7 of 9) of the animals sacrificed. Eighty-six percent (19 of 22) of the animals

necropsied between day 28 and day 32 after inoculation had severe ascites and larger tumors in the mesentery that extended to the pelvic cavity. In addition, 47% of the lymphoma-bearing mice (9 of 19) in the late ATL group had mild to marked white striations on the right ventricular epicardium (mineralization). Mild to marked splenomegaly was present in 35% (11 of 31) of the mice inoculated with ATL cells. The thymus was 5 to 10 times larger than controls in 2 of 22 mice in the late ATL group.

Histopathology

Microscopic evaluation (Table 1) of mice inoculated with ATL cells revealed lymphoma in the mesentery (26 of 31), thymus (20 of 31), liver (17 of 31), spleen (8 of 31), lungs (2 of 31), and bone marrow (5 of 31) in both early and late ATL groups. In the mesentery, spleen (Figure 1j), and thymus (Figure 1i), ATL cells formed large solid sheets or nodules replacing most of the normal tissue. In the liver, lymphoma was limited to the hilus region of the hepatic lobes, and was interpreted to be a direct extension of the mesenteric neoplasm. In the lungs, ATL cells formed large peribronchial cuffs (Figure 1h). Sixty-eight percent of the late ATL mice (13 of 19) had mild to marked mineralization in the epicardium of the right ventricle.

Plasma Concentrations of Calcium and PTHrP

Lymphoma-bearing mice in the late ATL group had a statistically significant ($P < 0.001$) increase in both ionized (6.7 ± 1.0 mg/dl) and total (15.8 ± 2.8 mg/dl) calcium concentrations compared to control mice (4.9 ± 0.2 and 8.7 ± 0.4 mg/dl, respectively) (Figure 2). Total plasma calcium concentrations were as great as 19.7 mg/dl in the SCID/bg mice engrafted with RV-ATL cells. A slight increase in calcium concentrations was observed in early ATL mice (day 20) when compared to age-matched control mice, although the difference was not statistically significant. Plasma PTHrP concentrations (Figure 3) were markedly increased in mice with lymphoma (12 to 140 pmol/L compared to <1.5 pmol/L in control mice). A strong positive correlation between plasma total calcium and PTHrP concentrations was present in SCID/bg mice engrafted with RV-ATL cells from both early ($r = 0.85$, $P < 0.001$) and late ATL ($r = 0.90$, $P < 0.001$) groups, respectively.

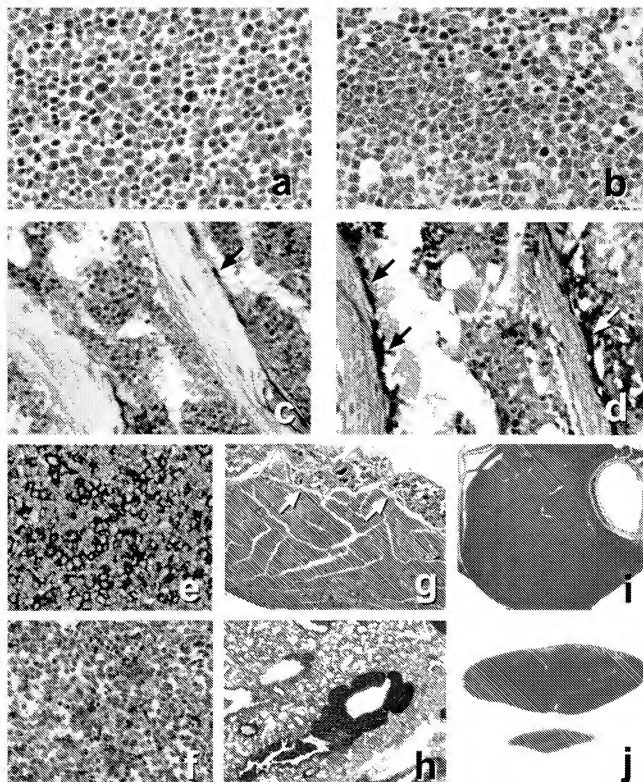


Figure 1. Immunohistochemistry, histology, and enzyme histochemistry of SCID/bg mice engrafted with human ATL cells. **a:** PTbP immunohistochemistry in mesenteric lymphoma (immunoperoxidase; original magnification, X590). **b:** Negative control for PTbP immunohistochemistry in mesenteric lymphoma (hematoxylin counterstain; original magnification, X590). **c:** Small osteoclasts (black arrow) in vertebrae from control mouse (tartrate-resistant acid phosphatase stain; original magnification X352). **d:** Markedly increased number of large osteoclasts (black and white arrows) in a SCID/bg mouse with ATL (day 32) (tartrate-resistant acid phosphatase stain; original magnification, X352). **e:** Positive CD45RO immunohistochemistry in RV-ATL cells engrafted in the mesentery of a SCID/bg mouse (immunoperoxidase; original magnification, X285). **f:** Negative CD45RO immunohistochemistry in the thymus of a control mouse (immunoperoxidase; original magnification, X285). **g:** Epicardial mineralization (white arrows) in a SCID/bg mouse with lymphoma and hypercalcemia (H&E stain; original magnification, X85). **h:** Peribronchial lymphoma in SCID/bg mouse (H&E stain; original magnification, X22). **i:** RV-ATL cells forming a nodular mass in the thymus of a SCID/bg mouse (H&E, original magnification, X22). **j:** Diffuse lymphoma in the spleen of a SCID/bg mouse (top) in comparison with a spleen from a control mouse (bottom) (H&E stain; original magnification, X8).

2224 Richard et al
 AJP June 2001, Vol. 158, No. 6

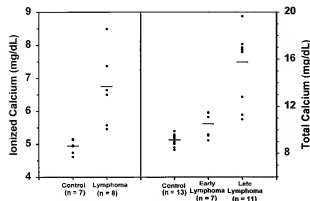


Figure 2. Plasma-ionized and total calcium concentrations (mg/dL). Mice engrafted with RV-ATL cells had significantly ($P < 0.001$) greater plasma-ionized calcium levels than controls (6.7 ± 1.0 versus 4.9 ± 0.2). Total calcium levels were also significantly ($P < 0.001$) higher in late lymphoma SCID-bg mice (15.8 ± 2.8) in comparison to age-matched controls (8.7 ± 0.4). Total calcium levels were not statistically different between early control (9.4 ± 0.3) and lymphoma (10.5 ± 1.0) mice. Bars indicate means.

PTHrP mRNA and Protein Expression by Xenografted RV-ATL Cells

Northern blot analysis revealed that RV-ATL cells forming mesenteric lymphoma in SCID/bg mice expressed PTHrP mRNA at very high levels (see Figure 5A). PTHrP mRNA expression in RV-ATL cells was greater than MT-2 cells, a well-established HTLV-1-infected cell line, that has been reported to secrete PTHrP⁴⁰ and was greater than SCC 2/88 cells, a squamous carcinoma cell line, that expresses PTHrP mRNA.⁴¹ No PTHrP mRNA was detectable by Northern blot analysis in normal human peripheral blood lymphocytes, Jurkat cells, and HT1-RV, a cell line immortalized *in vitro* by superinfection of RV-ATL cells with HTLV-1. PTHrP protein expression in RV-ATL cells xenografted in SCID/bg mice was evaluated by immunohistochemistry. The RV-ATL cells xenografted in the mesentery (Figure 1a), liver, spleen, and thymus of the lymphoma-bearing SCID/bg mice were strongly positive for PTHrP protein. PTHrP protein was identified both in the cytoplasm and the nucleus of the RV-ATL cells. The

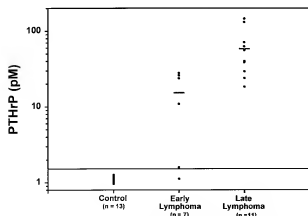


Figure 3. Plasma PTHrP (1-80) concentrations (pmol/L). Both early and late lymphoma mice had markedly increased circulating PTHrP levels (16.8 ± 11.9 and 61.3 ± 41.8 , respectively) in comparison to age-matched controls (<1.5). Bars indicate means.

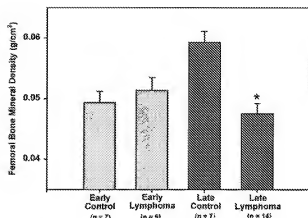


Figure 4. Femoral BMD (ng/cm²). BMD was significantly ($P < 0.001$) lower in late lymphoma SCID/bg mice (0.048 ± 0.006) in comparison to age-matched controls (0.059 ± 0.005). BMD was not statistically different between early control (0.049 ± 0.005) and lymphoma (0.051 ± 0.005) mice.

cytoplasmic:nuclear ratio of PTHrP immunohistochemical staining was ~4:1 based on histological examination of 200 positive RV-ATL cells. Other HTLV-1 cell lines were not examined immunohistochemically for PTHrP.

BMD and Histomorphometry

Femur BMD was significantly decreased ($P < 0.001$) in the late ATL group when compared with the control mice, whereas there was no significant difference observed between early ATL and control mice (Figure 4). Also, the late control group mice had a significant increase ($P < 0.01$) in BMD when compared to early control group mice. PTHrP concentrations and BMD were strongly negatively correlated ($r = -0.93$, $P < 0.005$). In addition, there was no significant difference (paired *t*-test, $P = 0.90$) in the BMD of the late ATL animals with bone marrow infiltration by lymphoma cells ($n = 5$) when compared to mice without bone marrow lymphoma ($n = 9$).

To examine osteoclastic bone resorption in this model, bone histomorphometric analysis was performed (Figure 6). Total bone volume (trabecular and cortical) was significantly decreased in both early ($P < 0.05$) and late ATL ($P < 0.001$) mice when compared to age-matched controls. There was no significant difference in percent trabecular bone volume. In contrast, osteoclastic bone resorption, percent osteoclast surface on trabeculae and number of osteoclasts/mm of trabecular bone, were increased two to fourfold in both early and late ATL-bearing SCID/bg mice in comparison to age-matched controls ($P < 0.001$).

Immunophenotypic Characterization of RV-ATL Cells

Analysis of cell surface antigens of the xenografted RV-ATL cells by flow cytometry revealed that CD4 and CD25 were expressed in 98 and 91% of the cells, respectively. CD3 and CD8 antigen expression was absent in 100% of the cells. Cell surface antigen expression in RV-ATL cells

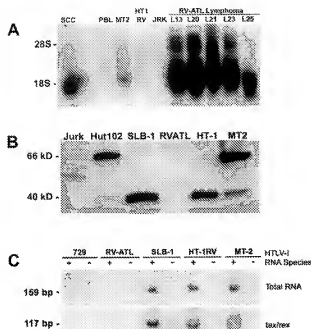


Figure 5. **A:** Northern blot showing PTHrP mRNA expression in xenografted lymphoma (RV-ATL) from five SCID/bg mice (L13 to L25) in the late ATL group in comparison to HT1-RV and MT-2 cells. Squamous cell carcinoma 2/88 line (SCC) served as positive control. Jurkat (Jk) and normal human peripheral blood lymphocytes served as negative controls. **B:** Western blot for HTLV-1 Tax expression. Tax (40 kD) was not detected in xenografted lymphoma (RVATL). In comparison, Tax was highly expressed in other HTLV-1-lymphoma cell lines tested (SLB-1, HT-1RV, and MT-2). Tax also was detected as a fusion protein with gp46 (66 kD) in HT102 and MT2 cells. Jurkat cells (Jurk) served as the negative control. **C:** RT-PCR for HTLV-1 mRNA expression in RV-ATL cells. **Top:** HTLV-1 total RNA (159 bp) was not detected in RV-ATL cells with 670/671 primers. HTLV-1 total RNA was detected in other HTLV-1 cell lines tested (SLB-1, HT-1RV, and MT-2). Negative control consisted of 729 B cells. **Bottom:** Doubly-spliced Tax/Rex (117 bp) also was not detected in RV-ATL cells with LA78/LA79 primers. Tax/Rex was detected in SLB-1, HT-1RV, and MT-2. Negative control consisted of 729 B cells.

was consistent with that observed in human patients with ATL, and indicates expansion of CD4⁺ cells expressing high levels of IL-2R α (CD25). Immunohistochemical phenotyping of xenografted RV-ATL cells demonstrated cell surface expression of CD45RO (an antigen expressed in most human thymocytes and mature activated T cells) in lymphoma of the mesentery (Figure 1e) and the thymus. Whereas there was absence of CD45RO expression in the spleen and thymus of control SCID/bg mice confirming the human origin of the lymphoma (Figure 1f).

HTLV-1 Provirus Copy Number and Tax Expression in Xenografted RV-ATL Cells

To accurately determine the number of integrated viral genomes in the RV-ATL and HT1-RV cell lines we performed quantitative real-time PCR. The RV-ATL line had 1.5 copies of provirus. The HT1-RV cell line, which was created by superinfection of the RV-ATL line with HTLV-1, had ~10 copies per cell. By Western blot analysis, RV-ATL cells did not express Tax (Figure 5B) or any structural HTLV-1 proteins, p19, p24, or gp46. Furthermore, tax/rax doubly spliced or total viral mRNA was not de-

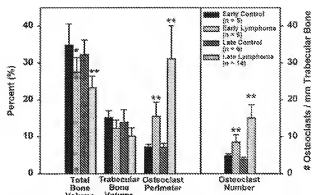


Figure 6. Histomorphometry of lumbar vertebrae in SCID/bg mice engrafted with RV-ATL cells (*, $P < 0.05$ different from age-matched control; **, $P < 0.001$ different from age-matched controls).

tected by RT-PCR (Figure 5C). The oligonucleotides primers, LA78/LA79 and 670/671, designed to amplify doubly spliced tax/rax RNA or all species of viral RNA, respectively, did not detect any expression of tax. In contrast, as demonstrated by Western blot analysis, HT1-RV, MT-2 and SLB-1 cells expressed high levels of Tax, p19, p24, and gp46. RT-PCR also demonstrated tax/rax mRNA in all three lines with the oligonucleotide primers.

Discussion

We report the establishment of a model of human T-cell lymphoma and HHM in SCID/bg mice with leukemic cells from a patient with HTLV-1-induced ATL. The primary ATL cells xenografted into SCID/bg mice maintained their morphological, immunohistochemical, and molecular features. In addition, xenografted ATL cells in SCID/bg mice induced hypercalcemia by secretion of PTHrP, which stimulated osteoclastic bone resorption.

SCID mice have been used as xenograft recipients for a variety of human neoplastic cells including hematological malignancies.^{32,42-46} The scid mutation was first described in 1983 in C.B-17 mice,⁴⁷ and is associated with a disrupted gene that encodes a DNA-dependent protein kinase, identified as *Prkdc*,⁴⁸ which is involved in immunoglobulin and T-cell receptor gene rearrangement. The mutation prevents T- and B-lymphocyte maturation. Despite this defect, engraftment of human hematological cells in SCID mice has been reported to be only modestly successful.^{49,50} NK-cell activity has been shown to play a key role in the rejection of human lymphoid neoplasms in SCID mice.^{31,51} We have previously used different methods to improve xenograft efficiency of ATL cells in SCID mice including γ -irradiation and administration of α -AGM₁ antiserum (anti-NK-cell antibodies).³¹ These methods were relatively efficient at reducing NK cell activity, but cumbersome to perform. To circumvent these difficulties we used SCID/bg mice in this investigation. As described, the beige mutation is responsible for a selective defect in the NK-cell immune response.⁵¹ When combined with the scid mutation, the beige mutation creates more favorable conditions for engraftment of human lym-

phoid neoplasms in mice, consequently allowing the establishment of a reliable animal model.

Most patients with ATL will develop hypercalcemia, which represents a life-threatening condition.¹⁵ PTHrP has been shown to be a causative factor of hypercalcemia in animals bearing human solid tumors.^{12,52} In ATL patients, PTHrP also has been identified as an important mediator of hypercalcemia, but other humoral factors such as IL-1 β and TGF- β have also been proposed to be involved.^{23,24} Previous studies examining the regulation of PTHrP in ATL cells were conducted *in vitro*, because of the lack of a reproducible animal model of ATL-associated HHM. Consequently, little is known about the specific mechanisms regulating PTHrP expression in ATL cells *in vivo*, and the interactions between PTHrP and other humoral factors in the development of hypercalcemia in humans with ATL.

A previous investigation³¹ by our laboratory reported the tumorigenic potential of ATL cells (RV-ATL) and HTLV-transformed cell lines (SLB-1 and JLB-1) by comparing the engraftment efficiency in SCID mice. In the present study, we examined and confirmed the central role played by PTHrP in the induction of hypercalcemia and increased osteoclastic resorption leading to bone loss in SCID/bg mice xenografted with ATL cells. An alternative model of HHM using SCID mice was reported by Takaori-Kondo and colleagues.⁵³ In contrast to our study, the authors showed increased levels of C-terminal PTHrP, but did not measure circulating levels of N-terminal PTHrP, the region of PTHrP that contains PTH-like activity, and lymphoma-bearing mice did not have increased osteoclastic bone resorption. Both increased circulating N-terminal PTHrP and excessive osteoclastic bone resorption are known to be important characteristic features of HHM observed in human patients with ATL.

PTHrP gene expression has been shown to be up-regulated by the HTLV-1 oncoprotein Tax *in vitro*.¹⁹ Dittmer and colleagues²⁰ have demonstrated that Tax interacts with the transcription factors Ets1 and Sp1 to transactivate the PTHrP P2 promoter when transfected in osteosarcoma OsA-CL cells. Tax can also mediate its effects on the PTHrP gene by the cellular transcription factors AP-1 and AP-2 as proposed by Prager and colleagues.⁵⁴ We were not able to detect Tax and other HTLV-1 viral proteins by Western blot analysis or HTLV-1 tax/rax mRNA by RT-PCR in ATL cells xenografted in SCID/bg. These data are consistent with the literature regarding the expression of HTLV-1 tax/rax mRNA in ATL cells from human patients.²¹ In contrast, HTLV-1 Tax protein and tax/rax mRNA were easily detectable in HT1-RV cells, a cell line produced by superinfection of RV-ATL cells with HTLV-1. Interestingly, no PTHrP mRNA was detectable by Northern blot analysis in HT1-RV cells. We determined the number of HTLV-1 proviral copy by quantitative real-time PCR using primers specifically designed to amplify the tax gene. We established that RV-ATL cells had 1.5 copies of provirus, and our results are consistent with published data for primary lymphocyte cell lines transformed by HTLV-1.⁵⁵ In contrast, the HT1-RV cells had 10 copies of HTLV-1 provirus. As we have previously reported, RV-ATL cells, as other ATL cell

lines, carry deletions of the provirus while retaining the tax/rax sequence.³²

Primary cell lines from human patients with ATL were shown to produce a wide variety of cytokines, including IL-2, TGF- β , TNF- β , and IL-1. IL-2 can up-regulate PTHrP gene expression in ATL cells by increasing its mRNA stability.²⁶ However, others have reported that IL-2 mRNA was not detectable by RT-PCR in ATL cells xenografted into SCID mice.⁵⁶ Our laboratory and others have previously demonstrated that TGF- β up-regulates PTHrP gene expression in multiple normal and neoplastic tissues,^{29,57-60} but there is no report addressing the effect of TGF- β on PTHrP secretion and expression in ATL cells. It will be useful to study the effect of TGF- β on both PTHrP gene transcription and mRNA stability *in vivo* using the SCID/bg model of human ATL.

Our laboratory has reported that PTHrP can bind to the human MT-2 cells (presumably by interacting with the PTH-1 receptor), and PTHrP inhibited cell growth of the MT-2 cells.⁶¹ Several reports have demonstrated that PTHrP modulates growth in different normal and cancer cells.⁶²⁻⁶⁷ The proliferative versus antiproliferative effects of PTHrP are dependent on the cell type. Falzon and Du⁶⁷ have shown that PTHrP had an antimetabolic effect in the human breast cancer cell line, MCF-7, using an autocrine/paracrine pathway mediated through the cell surface PTH-1 receptor. They also have shown that PTHrP exerted a mitogenic effect through the intracrine pathway, which correlated with nuclear accumulation of PTHrP. The exact mechanism by which nuclear localization of PTHrP stimulates cell growth is unknown, but nuclear PTHrP can inhibit apoptosis.⁶⁸ We examined the expression of the human PTH-1 receptor in four human ATL cell lines (RV-ATL, SLB-1, MT-2, and HT1-RV) by RT-PCR, and our data (not shown) demonstrated that the PTH-1 receptor was expressed in MT-2 cells (moderate), HT1-RV cells (low), and SLB-1 cells (very low), but was not expressed in RV-ATL cells. Interestingly, immunohistochemistry was strongly positive for PTHrP both in the cytoplasm and nucleus of RV-ATL cells xenografted into SCID/bg mice. The exact role of nuclear PTHrP on regulation of proliferation or apoptosis in ATL cells remains to be determined both *in vitro* and *in vivo*. Our model will enable us to examine the effect of PTHrP on ATL cell growth *in vivo*.

In conclusion, the SCID/bg mouse model of human ATL will be useful to study the regulation of PTHrP and the interrelationships between PTHrP and cytokines produced by ATL cells in the induction of HHM *in vivo*. The model is reproducible and mimics the disease observed in human patients with ATL. In addition, the model will be useful to develop new therapeutic strategies for the treatment of hypercalcemia observed in patients with ATL.

Acknowledgments

We thank Alan D. Flechtner and Anne-Evelyn Handley for their excellent technical assistance.

References

- Rosol TJ, Capen CC: Mechanisms of cancer-induced hypercalcemia. *Lab Invest* 1992, 67:690-702
- Rosol TJ, Capen CC: Pathogenesis of humoral hypercalcemia of malignancy. *Domest Anim Endocrinol* 1988, 5:1-21
- Broadus AE, Mangin M, Ikeda K, Insogna KL, Weir EC, Burris WJ, Stewart AF: Humoral hypercalcemia of cancer: Identification of a novel parathyroid hormone-like peptide. *N Engl J Med* 1988, 319: 556-563
- Suva LJ, Winslow GA, Wettenhall HE, Hammonds HG, Moseley JM, Dielenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, Chen EY: A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 1987, 237:893-896
- Strewler GJ, Stern PH, Jacobs JW, Evelyn J, Klein RF, Leung SC, Rosenblatt M, Nissenson HA: Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. *J Clin Invest* 1987, 80:1903-1907
- Burris WJ, Wu T, Bunch C, Wyslowski J, Insogna KL, Weir EC, Broadus AE, Stewart AF: Identification of a novel 17,000-dalton parathyroid hormone-like adenylyl cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *J Biol Chem* 1987, 262:715-7156
- Hornich N, Caulfield MP, Fisher JE, Goldman ME, McKee RL, Hoeggen JE, Levy JJ, Nutt HF, Rodda SB, Schofield TL: Similarity of synthetic peptide from human tumor to parathyroid hormone in vivo and in vitro. *Science* 1987, 238:1566-1568
- Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall HE, Stapleton D, Dielenbach-Jagger H, Ure F, Michelangeli VP, Simmons HA: Parathyroid hormone-related protein of malignancy: active synthetic fragments. *Science* 1987, 238:1568-1570
- Stewart AF, Mangin M, Wu T, Goumas D, Insogna KL, Burris WJ, Broadus AE: Synthetic human parathyroid hormone-like protein stimulates bone resorption and causes hypercalcemia in rats. *J Clin Invest* 1988, 81:566-600
- Roodman GD: Mechanisms of bone lesions in multiple myeloma and lymphoma. *Cancer* 1997, 80:1557-1563
- Ikeda K, Mangin M, Dreyer BE, Webb AC, Posillico JT, Stewart AF, Bender NH, Weir EC, Insogna KL, Broadus AE: Identification of transcripts encoding a parathyroid hormone-like peptide in messenger RNAs from a variety of human and animal tumors associated with humoral hypercalcemia of malignancy. *J Clin Invest* 1988, 81:2010-2014
- Kukreja SC, Shewin DH, Wimbiscus SA, Ebeling PR, Danks JA, Rodda CP, Wood WJ, Martin TJ: Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy-associated hypercalcemia due to human tumors. *J Clin Invest* 1989, 82:1798-1802
- Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H: Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* 1977, 50:481-492
- Polesz BJ, Russett FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1980, 77:7415-7419
- Bunn JR, Polesz FW, Jaffe E, Blayney D, Young RC, Matthews MJ, Blattner W, Broder S, Robert-Guroff M, Gallo RC: Clinical course of retrovirus-associated adult T-cell lymphoma in the United States. *N Engl J Med* 1983, 309:257-264
- Koralnik JJ, Gossain A, Klotman ME, Lo MA, Bornemann ZN, Franchini G: Protein isoforms encoded by the pX region of human T-cell leukemia/lymphotropic virus type I. *Proc Natl Acad Sci USA* 1992, 89: 8813-8817
- Seki M, Hattori S, Hirayama Y, Yoshida M: Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc Natl Acad Sci USA* 1983, 80:3618-3622
- Mosnar JM, Devaux C: Multiple control levels of cell proliferation by human T-cell leukemia virus type 1 Tax protein. *Virology* 1999, 257: 277-284
- Watanabe T, Yamaguchi K, Takatsuki K, Osame M, Yoshida M: Constitutive expression of parathyroid hormone-related protein gene in human T-cell leukemia virus type 1 (HTLV-1) carriers and adult T-cell leukemia patients that can be trans-activated by HTLV-1 tax gene. *J Exp Med* 1990, 172:759-765
- Dittmer J, Pise-Masison CA, Glomens KE, Choi KS, Brady JN: Interaction of human T-cell lymphotropic virus type 1 Tax, Ets1, and Sp1 in transactivation of the PTHrP P2 promoter. *J Biol Chem* 1997, 272: 4953-4958
- Franchini G, Wong-Staal F, Gallo RC: Human T-cell leukemia virus (HTLV-1) transcripts in fresh and cultured cells of patients with adult T-cell leukemia. *Proc Natl Acad Sci USA* 1984, 81:6207-6211
- Kinoshita I, Shimoyama M, Tobinai K, Ito M, Ikeda S, Tajima K, Shimotohno K, Sugimura T: Detection of mRNA for the tax/rox1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc Natl Acad Sci USA* 1989, 86:5620-5624
- Wano Y, Hattori T, Matsutaka M, Takatsuki K, Chua AO, Gubler U, Greene WC: Interleukin 1 gene expression in adult T-cell leukemia. *J Clin Invest* 1987, 80:911-916
- Nitsky Y, Urushizaki Y, Koshida Y, Terui K, Matsura K, Kohno Y, Urushizaki T: Expression of TGF- β gene in adult T-cell leukemia. *Blood* 1989, 71:263-266
- Ishibashi K, Ishitsuka K, Chuman Y, Otsuka M, Kuwazuru Y, Iwahashi M, Utsunomiya A, Hanada S, Sakurai T, Arima T: Tumor necrosis factor- β in the serum of adult T-cell leukemia with hypercalcemia. *Blood* 1991, 77:2451-2455
- Ikeda K, Okazaki H, Inoue D, Ohno H, Ogata E, Matsumoto T: Interleukin-2 increases production and secretion of parathyroid hormone-related peptide by human T-cell leukemia virus type I-infected T cells: possible role in hypercalcemia associated with adult T-cell leukemia. *Endocrinology* 1993, 132:2551-2556
- Mori N, Ohtsuki K, Murakami S, Wake A, Shirakawa F, Morimoto I, Oda S, Ito S: Enhancing effect of interleukin-2 on production of parathyroid hormone-related protein by adult T-cell leukemia cells. *Jpn J Cancer Res* 1993, 84:425-430
- Ohnori M, Nagai M, Fujita M, Dobashi H, Taseika T, Yamaoka G, Kawanishi K, Tanikawa M, Takahara J: A novel mature B-cell line (DOB1-6) producing both parathyroid hormone-related protein and interleukin-6 from a myeloma patient presenting with hypercalcemia. *Br J Haematol* 1998, 101:686-693
- Merrymann J, DeWille JW, Workmeister JH, Capen CC, Rosol TJ: Effects of transforming growth factor- β on parathyroid hormone-related protein production and ribonucleic acid expression by a squamous carcinoma cell line in vitro. *Endocrinology* 1994, 134:2424-2430
- Funk JL, Krul EJ, Moser AH, Shigenaga JK, Strewler GJ, Grunfeld C, Feingold KR: Endotoxin increases parathyroid hormone-related protein mRNA levels in mouse spleen. Mediation by tumor necrosis factor. *J Clin Invest* 1993, 92:2546-2552
- Fouer G, Stewart SA, Baird SM, Lee F, Feuer R, Chen IS: Potential role of natural killer cells in controlling lymphomagenesis by human T-cell leukemia viruses. *J Virol* 1995, 69:1328-1333
- Fouer G, Zack JA, Harrington JR, Waldenstrom R, Rosenblatt JD, Wachsmen W, Baird SM, Chen IS: Establishment of human T-cell leukemia virus type I T-cell lymphomas in severe combined immunodeficient mice. *Blood* 1993, 82:722-731
- Weckmann MT, Grone A, Capen CC, Rosol TJ: Regulation of parathyroid hormone-related protein secretion and mRNA expression in normal human keratinocytes and a squamous carcinoma cell line. *Exp Cell Res* 1997, 232:79-89
- Ehrlich LS, Krausslich HG, Wimmer E, Carlier CA: Expression in *Escherichia coli* and purification of human immunodeficiency virus type 1 capsid protein (p24). *AIDS Res Hum Retroviruses* 1990, 6:1169-1175
- Kinsella JT, Wong FH, Wang JJ, Ratner L: Construction and characterization of infectious human T-cell leukemia virus type 1 molecular clones. *Virology* 1994, 204:656-664
- Albrecht B, Collins ND, Newbound GC, Ratner L, Lairmore MD: Quantification of human T-cell lymphotropic virus type 1 proviral load by quantitative competitive polymerase chain reaction. *J Virol Methods* 1998, 75:123-140
- Palko TJ, Tanner ME, Socorro FM, Streilein DF, Clark ME, Haynes BF: Mapping of immunogenic regions of human T-cell leukemia virus type 1 (HTLV-1) gp46 and gp21 envelope glycoproteins with anti-

- encoded synthetic peptides and a monoclonal antibody to gp46. *J Immunol* 1989, 142:971-978
38. Chomczynski P: A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biochemical Sciences* 1993, 15:532-537
 39. Kusuhara K, Anderson M, Pettiford SM, Green PL: Human T-cell leukemia virus type 2 Rex protein increases stability and promotes nuclear to cytoplasmic transport of gag/pol and env RNAs. *J Virol* 1999, 73:8112-8119
 40. Fukumoto S, Matsumoto T, Watanabe T, Takahashi H, Miyoshi I, Ogata E: Secretion of parathyroid hormone-like activity from human T-cell lymphotropic virus type I-infected lymphocytes. *Cancer Res* 1989, 49:3849-3852
 41. Gröne A, Weckmann MT, Capen CC, Rosol TJ: Regulation of parathyroid hormone-related protein expression in a canine squamous carcinoma cell line by colchicine. *Exp Toxicol Pathol* 1996, 50:365-370
 42. Itoh T, Shiota M, Takanashi M, Hojo I, Satoh H, Matsuzawa A, Moriama T, Watanabe T, Hirai K, Mori S: Engraftment of human non-Hodgkin lymphomas in mice with severe combined immunodeficiency. *Cancer* 1993, 72:2696-2694
 43. Visoneau S, Cesano A, Torosian HM, Miller EJ, Santoli D: Growth characteristics and metastatic properties of human breast cancer xenografts in immunodeficient mice. *Am J Pathol* 1986, 152:1299-1311
 44. Nemeth JA, Herb JF, Barroso U, Ho Z, Grignon DJ, Cher ML: Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. *Cancer Res* 1990, 50:1987-1993
 45. Angevin E, Glukhova L, Pavon C, Chassevante A, Terrier-Lacombe MJ, Goguel AF, Bougaran J, Ardouin P, Court BH, Perrin JL, Vallancien G, Triebel F, Escudier B: Human renal cell carcinoma xenografts in SCID mice: tumorigenicity correlates with a poor clinical prognosis. *Lab Invest* 1999, 79:879-888
 46. Xu Y, Silver DF, Yang NP, Ollazoglu E, Hempling RE, Piver MS, Repasky EA: Characterization of human ovarian carcinomas in a SCID mouse model. *Gynecol Oncol* 1999, 72:161-170
 47. Bosma GC, Custer RP, Bosma MJ: A severe combined immunodeficiency mutation in the mouse. *Nature* 1983, 301:527-530
 48. Kirchgesner CU, Patel CK, Evans JW, Cuomo CA, Fried LM, Carter T, Dettlinger MA, Brown JM: DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science* 1995, 267:1178-1183
 49. Waller EK, Kamel OW, Cleary ML, Majumdar AS, Schick MR, Lieberman M, Weissman IL: Growth of primary T-cell non-Hodgkin's lymphoma in SCID-hu mice: requirement for a human lymphoid microenvironment. *Blood* 1991, 78:2650-2665
 50. Blondi A, Motta T, Garofalo A, Rossi V, Giudici G, Rizzo V, Pitolli P, Corneo G, Barbul T, Parma A: Human T-cell lymphoblastic lymphoma expressing the T-cell receptor gamma/delta established in immunodeficient (bg/nxid) mice. *Leukemia* 1993, 7:281-289
 51. Christianson SW, Greiner DL, Schweitzer IB, Gott B, Beamer GL, Schweitzer PA, Hesselton RM, Shultz LD: Role of natural killer cells on engraftment of human lymphoid cells and on metastasis of human T-lymphoblastic leukemia cells in C57BL/6J-scid mice and in C57BL/6J-scid by mice. *Cell Immunol* 1996, 171:186-199
 52. Kukreja SC, Rosol TJ, Wimbush SA, Shewin DH, Gril V, Barengolis EJ, Martin TJ: Tumor resection and antibodies to parathyroid hormone-related protein cause similar changes on bone histomorphometry in hypercalcemia of cancer. *Endocrinology* 1990, 127:305-310
 53. Takatori-Kondo A, Imada K, Yamamoto I, Kunitomi A, Numata Y, Sawada H, Uchiyama T: Parathyroid hormone-related protein-induced hypercalcemia in SCID mice engrafted with adult T-cell leukemia cells. *Blood* 1998, 91:4747-4751
 54. Prager D, Rosenblatt JD, Ejima E: Hypercalcemia, parathyroid hormone-related protein expression and human T-cell leukemia virus infection. *Leuk Lymphoma* 1994, 14:395-400
 55. Albrecht B, Collins ND, Burniston MT, Nisbet JW, Ratner L, Green PL, Altmire MD: Human T-lymphotropic virus type 1 open reading frame 1p12(0) is required for efficient viral infectivity in primary lymphocytes. *J Virol* 2000, 74:9986-9995
 56. Imada K, Takatori-Kondo A, Sawada H, Imura A, Kawamata S, Okuma M, Uchiyama T: Serial transplantation of adult T-cell leukemia cells into severe combined immunodeficient mice. *Jpn J Cancer Res* 1996, 87:887-892
 57. Yasui T, Uemura H, Irahara M, Aono T: Effects of transforming growth factor- β on the production of parathyroid hormone-related peptide in a human ovarian cancer cell line in vitro. *J Obstet Gynaecol Res* 1997, 23:231-238
 58. Werkmeister JF, Blomme EA, Weckmann MT, Grone A, McCauley LK, Wade AB, O'Rourke J, Capen CC, Rosol TJ: Effect of transforming growth factor- β 1 on parathyroid hormone-related protein secretion and mRNA expression by normal human keratinocytes in vitro. *Endocrinology* 1998, 82:291-299
 59. Yin JJ, Selander K, Chargin JM, Dallas M, Grubbs BG, Wiser R, Messague J, Murphy GR, Guise TA: TGF- β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999, 103:197-206
 60. Benitez-Verguizas J, Loarte D, De Miguel F, Esbrit P: Effects of transforming growth factor β 1 on cell growth and parathyroid hormone-related protein in Walker 256 tumor cells. *Life Sci* 1999, 65:1807-1816
 61. McCauley LK, Rosol TJ, Merryman JL, Capen CC: Parathyroid hormone-related protein binding to human T-cell lymphotropic virus type I-infected lymphocytes. *Endocrinology* 1992, 130:300-306
 62. Hamura M, Abrahamson PA, Foss KA, Wu G, Cockett AT, Delfos LJ: Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. *Urology* 1994, 43:675-679
 63. Raibani SA, Glesby J, Liu B, Goltzman D: Regulation in vivo of the growth of Leydig cell tumors by antisense ribonucleic acid for parathyroid hormone-related peptide. *Endocrinology* 1995, 136:5416-5422
 64. Burton PB, Moniz C, Knight DE: Parathyroid hormone-related peptide can function as an autocrine growth factor in human renal cell carcinoma. *Biochem Biophys Res Commun* 1990, 167:1134-1138
 65. Massfelder T, Dann P, Wu TL, Vasavada R, Helwig JJ, Stewart AF: Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. *Proc Natl Acad Sci USA* 1997, 94:13630-13635
 66. Kano J, Sugimoto T, Fukase M, Fujita T: The activation of cAMP-dependent protein kinase is directly linked to the inhibition of osteoblast proliferation (UMF-106) by parathyroid hormone-related protein. *Biochem Biophys Res Commun* 1991, 179:97-101
 67. Fabron M, Du P: Enhanced growth of MCF-7 breast cancer cells overexpressing parathyroid hormone-related peptide. *Endocrinology* 2000, 141:1892-1892
 68. Dougherty KM, Blomme EA, Koh AJ, Henderson JE, Pienta KJ, Rosol TJ, McCauley LK: Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res* 1999, 59:6015-6022

PARATHYROID HORMONE RELATED PEPTIDE CAN FUNCTION AS AN AUTOCRINE GROWTH FACTOR IN HUMAN RENAL CELL CARCINOMA

PAUL B. J. BURTON¹, CAJE MONIZ² AND DEREK E. KNIGHT¹

¹Division of Biomedical Sciences, Kings College, London WC2R 2LS, United Kingdom

²Dept. of Chemical Pathology, Kings College Hospital, London SE5 9RS, United Kingdom

Received February 12, 1990

SUMMARY: Parathyroid hormone related peptide (PTHrP) has been implicated in the cause of the hypercalcemia associated with a number of malignant tumours. The data presented here suggests that PTHrP (in addition to its known role of mediating hypercalcemia) may be involved in the autocrine regulation of growth of some tumours. Polyclonal PTHrP antiserum almost totally inhibited the growth of a human renal cell carcinoma cell line, known to secrete PTHrP, *in vitro* and growth was significantly inhibited by the competitive PTH antagonist PTH (3-34)NH₂.

© 1990 Academic Press, Inc.

The initiation and perpetuation of carcinogenesis is a multifactorial process (1). While the ectopic and unregulated secretion of hormones frequently complicate the clinical management of a number of malignancies, the role they play in the autocrine regulation of tumour growth is becoming increasingly apparent (2). Throughout the last decade there has been much interest in the syndrome of humoral hypercalcemia of malignancy (SHHM). SHHM is associated with some 20% of tumours, most commonly renal cell and lung small cell carcinoma, and is characterised by raised serum levels of calcium, increased urinary excretion of cAMP and phosphaturia. Serum levels of bioactive parathyroid hormone (PTH) are found to be elevated, yet paradoxically serum levels of immunoreactive PTH are found to be normal or even suppressed (3). This suggests the presence of a circulating factor with PTH like bioactivity but which is structurally and immunologically distinct from it.

Recently, a peptide has been isolated from a variety of hypercalcemic tumours, which is composed of 141 amino acids, of which 8 out of the first 13 are common to PTH. This limited N-terminal amino acid homology with PTH is sufficient to confer upon the peptide PTH like bioactivity and has led to it being termed Parathyroid hormone related peptide or PTHrP (4). While PTHrP is now believed to be a major contributing factor to the aetiology of SHHM, the aim of this study was to determine

TION AS
CELL

NIGHT

ted Kingdom

ited Kingdom

has been
umber of
PTHrP (in
involved
olyclonal
an renal
1 growth
PTH (3-

1). While
clinical
regulation
st decade
alignancy
renal cell
calcium,
bioactive
levels of
gests the
rally and

umours,
nmon to
o confer
athyroid
a major
termine

whether or not the protein plays any part in the autocrine regulation of growth of a hypercalcemic renal carcinoma cell line known to secrete PTHrP.

MATERIALS AND METHODS

Materials: PTHrP (1-34) was purchased from Peninsula Laboratories (Merseyside, UK). PTH (1-34) and PTH (3-34)NH₂ were obtained from Sigma. PTHrP (1-141) was kindly donated by Dr. R. G. Hammonds (Genentech, Inc, Calif, U.S.A.). Polyclonal PTHrP antiserum raised against PTHrP (1-34) was the generous gift of Drs. G. V. Segre and H. Jueppner (Massachusetts General Hospital, Boston, Mass).

Cell Culture: SKRC-1 cells (kindly supplied by Dr. N. H. Bander, New York, U.S.A.), were grown as previously described (5), with minor modifications, substituting DMEM for Eagles MEM. In all experiments cells were seeded into 24 well plates (Nunc) and grown in medium containing 10% FCS. The cells were fed daily for the desired time period with test substance (either peptide or antiserum), trypsinised and counted using an Elzone particle counter model 280PC. Data are expressed as mean \pm S.E.M. and statistical significance was determined by Students t-test, a value of $P_0 < 0.01$ being taken as statistically significant.

RESULTS

The approach used to investigate the hypothesis that PTHrP might function as an autocrine growth factor in some tumours was to selectively add or remove the peptide from growing cultures of SKRC-1 cells. These are known to secrete PTHrP *in vitro* and produce hypercalcemia when implanted into nude mice *in vivo* (5).

Cells were treated for 3 days with PTH (1-34), PTHrP (1-34) or PTHrP (1-141) over a

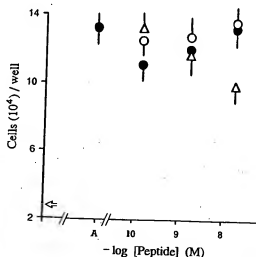


Fig. 1. Effect of PTH and PTHrP analogues on growth of SKRC-1 cells *in vitro*.

Cells were seeded at a density of 2.5×10^4 cells per well (shown by arrow). After 12 hours cells were fed with medium alone (shown by point A) or containing either PTH (1-34) (●), PTHrP (1-34) (○), or PTHrP (1-141) (△), at the concentration indicated. Cells were fed daily and after 3 days were trypsinised and counted using an Elzone particle counter. (Points represent the mean \pm S.E.M; n=3.)

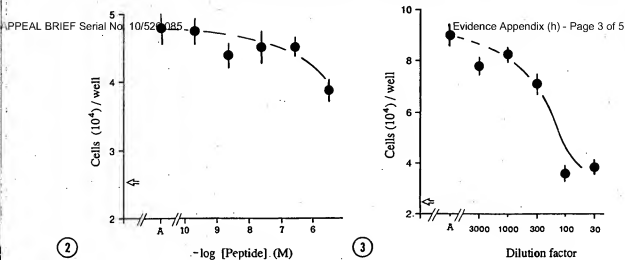


Fig. 2. Effect of the competitive PTH receptor antagonist PTH (3-34)NH₂ on the growth of SKRC-1 cells in vitro.

Cells were seeded at a concentration of 2.5×10^4 cells per well (shown by arrow). After 12 hours the cells were fed with medium alone (shown by point A) or containing PTH (3-34)NH₂ at the concentrations indicated. After a further 24 hours the cells were trypsinised and counted using an Elzone particle counter. (Points represent the mean \pm S.E.M.; $n=4$.)

Fig. 3. Effect of PTHrP antiserum on the growth of SKRC-1 cells in vitro.

Cells were seeded at a density of 2.5×10^4 cells per well (shown by arrow). After 12 hours cells were fed with medium alone (shown by point A) or containing polyclonal PTHrP antiserum diluted as indicated. Cells were fed after 24 hours. After a further 24 hours cells were trypsinised and counted using an Elzone particle counter. (Points represent the mean \pm S.E.M.; $n=4$.)

Table 1. Effect of PTHrP antiserum on ROS 17/2.8 cells, bovine adrenal chromaffin cells and SKRC-1 cells

	TREATMENT		
	NONE	PTHrP ANTISERUM	P _o
ROS 17/2.8	46,238 (2468)	44,850 (2235)	N.S.
B.A.C.	616,818 (50,640)	677,454 (59,349)	N.S.
SKRC-1	90,460 (6027)	35,875 (1276)	<0.01

ROS 17/2.8 cells were seeded at a density of 2.5×10^4 cells per well. After 12 hours the cells were fed with medium containing PTHrP antiserum diluted 1:100. The cells were fed again after 24 hours and after a further 24 hours cells were trypsinised and counted. A similar protocol was employed for the bovine adrenal chromaffin cells (BAC) and SKRC-1 cells, except that these were seeded at a density of 6×10^5 and 2.5×10^4 cells per well, respectively. In the case of the bovine adrenal chromaffin cells, they were left for 4 days before being treated with medium containing PTHrP antiserum. (Data represents the mean (S.E.M.); $n=4$. N.S. represents no significant difference between groups).

concentration range of 0.125 - 12.5nM (Fig. 1). The effect of exogenously added peptide was found to be mild, with 12.5nM PTHrP (1-141) appearing to inhibit growth by about 15%. This lack of effect could result from the cells secreting sufficient PTHrP to mask the mitogenic effect of added peptide.

To investigate whether secreted PTHrP exerted any effect on growth through a PTH type receptor cells were treated with the competitive PTH receptor antagonist, PTH (3-34)NH₂. After 24 hours this PTH analogue, at a concentration of 1.25μM, was found to significantly inhibit cell growth (Fig. 2).

Addition of polyclonal PTHrP antiserum at a dilution of 1:100 almost totally inhibited the growth of SKRC-1 cells (Fig. 3). This effect was specific for these cells since it had no effect on the growth of ROS 17/2.8 cells (which possess PTH receptors) nor was it cytotoxic when tested on quiescent cultures of bovine adrenal medullary cells (Table 1).

DISCUSSION

The regulation of normal cellular growth and proliferation is complex and far from understood. Normal cells on their way to becoming cancer cells pass through several stages during which they loose their requirement for certain growth factors and instead pass uncontrollably through the cell cycle (6).

The hypercalcemic factor PTHrP, has been isolated from a number of human and animal tumours (7) and also from the culture medium of human keratinocytes (8). On addition of PTHrP to human keratinocytes, these cells undergo terminal differentiation and cease proliferation (9), furthermore, the peptide has been reported to play a role in the differentiation of F9 teratocarcinoma derived stem cells (10). It would appear, therefore, that PTHrP does have the ability to modulate cellular development. The data we present here would suggest that PTHrP is involved in the regulation of growth of the renal cell carcinoma cell line SKRC-1. While PTHrP is not expressed by normal adult kidney, it is present in the developing tubules of human fetal kidney (11). Its re-expression in the transformed phenotype of the adult is similar to the appearance of alpha feto-protein in primary hepatocellular carcinoma which is only present in the fetal liver. The data presented here would further support the hypothesis that PTHrP could be a fetal growth factor.

It is possible that the ability of PTHrP (1-141) to inhibit the growth of SKRC-1 cells results from the full length amino acid sequence undergoing proteolytic cleavage to yield fragments with direct inhibitory activity or which have receptor antagonistic activity. Certainly different portions of mitogens are known to exert different biological effects (12). Alternatively it is possible that the full length peptide (or a cleaved fragment) may act synergistically with other mitogens, such as TGF-beta, present at high concentration in the medium containing 10% serum, to induce its

effects. The exact reason is unclear. The high concentration of the PTH receptor antagonist PTH (3-34)NH₂ required to inhibit growth over 24 hours, can be explained in terms of the ratio of antagonist to agonist required for it to produce its effect. PTH (3-34)NH₂ must be present at between a 100 and a 1000 fold molar excess in order to have antagonistic activity (5).

The PTHrP antiserum used in this study is specific for PTHrP and was not found to be cytotoxic to other cell lines. Antiserum had no effect on ROS 17/2.8 cells which do not synthesize or secrete PTHrP but do possess PTH receptors. Inhibition of growth of SKRC-1 cells by "immuno-neutralising" endogenously secreted PTHrP might suggest the use of antiserum directed against PTHrP in the therapy of SHHM, reducing both hypercalcemia and tumour growth.

These data support the hypothesis that PTHrP contributes to the autocrine growth of a hypercalcemic renal cell carcinoma. This appears to be mediated via a PTH receptor and growth can be inhibited by the use of a polyclonal PTHrP antiserum, or a PTH receptor antagonist. The role PTHrP plays in the growth of other "hypercalcemic" tumours (especially those that do not possess PTH receptors) is yet to be investigated.

ACKNOWLEDGMENT: This work was supported by the Nuffield Foundation.

REFERENCES

- Farber, E. (1984) Cancer Res. 44: 4217-4223.
- Sporn, M.B. and Todaro, G.J. (1980) N. Eng. J. Med. 303: 878-880.
- Stewart, A.F., Horst, R., Defetos, L.J., Cadman, E.C., Lang, R. and Broadus, A.E. (1980) N. Eng. J. Med. 303: 1377-1381.
- Martin T.J. and Suva L.J. (1989). *Ballier's Clin. Endo. Metab.* 2(4) 1003-1029.
- Weir, E.C., Insogna, K.L., Brownstein, D.G., Bander, N.H. and Broadus, A.E. (1988) *J. Clin. Invest.* 81: 818-821.
- Yuspa, S.H. (1981) In: *Models, Mechanisms and Etiology of Tumour Promotion*. M. Borzsonyi, K. Lapis, N.E. Day, and H. Yamasaki eds. IARC Scientific Publications, Lyon. No. 56, pp191-204.
- Ikeda, K., Weir, E.C., Mangin, M., Dannies, P.S., Kinder, B., Defetos, L.J., Brown, E.M. and Broadus, A.E. (1988). *Mol. Endocrinol.* 2, 1230-1236.
- Meredino, J.J. Jr., Insogna, K.L., Milstone, L.M., Broadus, A.E. and Stewart, A.F. (1986). *Science*. 231: 388-390.
- Holick, M.F., Nussbaum, S., and Persons, K.S. 10th Annual Meeting of the American Society of Bone and Mineral Research, New Orleans, LA. 1988. p214 (Abstract 582).
- Nissenson, R.A., King, K., Diep, D., Bruce, R. and Stewler, G.J. Joint meeting of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, Calif. 1989, p482a (Abstract 2714).
- Burton, P.B.J., Moniz, C., Quirke, P., Tzannatos, C., Pickles, A., Dixit, M., Triffitt, J.T., Jueppner, H., Segre, G.V. and Knight, D.E. (1990) *Mol. Cell Endocrinol.* (in press).
- Weber, S., Zuckerman, J.E., Bostwick, D.G., Bensch, K.G., Branimir, I.S. and Raffin, T.A. (1985) *J. Clin. Invest.* 75: 306-309.



(19)

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 1 197 225 A1

(12)

EUROPEAN PATENT APPLICATION
published in accordance with Art. 158(3) EPC

(43) Date of publication:

17.04.2002 Bulletin 2002/16

(51) Int Cl.⁷: **A61K 45/00**, A61K 39/395,
A61P 3/14, A61P 29/00,
A61P 37/02

(21) Application number: **00942455.7**

(86) International application number:

PCT/JP00/04414(22) Date of filing: **03.07.2000**

(87) International publication number:

WO 01/02011 (11.01.2001 Gazette 2001/02)

(84) Designated Contracting States:

**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE**

Designated Extension States:

AL LT LV MK RO SI• **ONUMA, Etsuro,**Chugai Selyaku Kabushiki Kaisha
Gotenba-shi, Shizuoka 412-8513 (JP)• **TSUNEMARI, Toshiaki,**Chugai Selyaku K. Kaisha
Gotenba-shi, Shizuoka 412-8513 (JP)• **SAITO, Hidemi,**Chugai Selyaku Kabushiki Kaisha
Gotenba-shi, Shizuoka 412-8513 (JP)• **AZUMA, Yumiko,**Chugai Selyaku Kabushiki Kaisha
Gotenba-shi, Shizuoka 412-8513 (JP)(30) Priority: **02.07.1999 JP 18979399**(71) Applicant: **CHUGAI SEIYAKU KABUSHIKI
KAISHA**

Tokyo, 115-8543 (JP)

(74) Representative: **Maschio, Antonio et al**D Young & Co.,
21 New Fetter Lane
London EC4A 1DA (GB)

(72) Inventors:

• **OGATA, Etsuro**

Setagaya-ku, Tokyo 157-0076 (JP)

• **SATO, Koh, Chugai Selyaku Kabushiki Kaisha**
Chuo-ku, Tokyo 104-8301 (JP)(54) **REMEDIES FOR DISEASES CAUSED BY PTH OR PTHrP**

(57) The present invention is directed to providing a therapeutic agent for diseases caused by PTH or PTHrP. The present invention provides a therapeutic agent for diseases caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antag-

onist binding to a PTH receptor or PTHrP receptor, or a substance which binds to a ligand of such a receptor to thereby promote or inhibit binding of the ligand to the receptor.

EP 1 197 225 A1

EP 1 197 225 A1

Description

TECHNICAL FIELD

- 5 [0001] The present invention relates to a therapeutic agent for diseases caused by parathyroid hormone (PTH) or parathyroid hormone-related protein (PTHrP).

BACKGROUND ART

- 10 [0002] A parathyroid hormone-related protein (PTHrP) is a protein, which was identified in 1987 by investigation of humoral factors causing humoral hypercalcemia of malignancy. It is known that the N-terminus of the protein expresses functions thereof by binding to a receptor (PTH/PTHrP receptor) which is common to parathyroid hormone (PTH).

- [0003] It has been reported that PTHrP is generated from various tumor tissues, but it is also generated from a wide range of normal tissues such as skin, mammary gland, uterus, placenta, bone, smooth muscle, heart, lung, kidney, liver and brain, and exhibits various functions locally via autocrine/paracrine secretory mechanism.

- 15 [0004] A PTH/PTHrP receptor strongly expresses in kidney and bone, a target organ of PTH and PTHrP. However, it has been clarified that, apart from these organs, this receptor expresses also in aorta, adrenal, brain, mammary gland, heart, digestive tract, liver, lung, skeletal muscle, ovary, placenta, skin, stomach and uterus etc., and that the receptor shows an extremely similar distribution to PTHrP.

- 20 [0005] As functions of PTHrP, there are known not only a bone resorption promoting function by activation of osteoclasts in bone and a calcium reabsorption promoting function by acting to distal convoluted tubules of nephros, but also the following (1) to (3):

- 25 (1) Involvement in calcium transportation system (e.g. mammary gland epithelium, placenta etc.) in epidermal cells,
(2) A strong smooth muscle relaxing activity (e.g. uterus, urinary tract, blood vessel and digestive tract etc.), and
(3) Involvement in growth, differentiation and development,

but the physiological role of PTHrP in many tissues other than those stated above is still unknown.

- 30 [0006] For example, PTHrP and a PTH/PTHrP receptor express in the central nervous system (CNS), but there has been hardly any clarification of the functions thereof. When localization of PTHrP mRNA in rat brain was analyzed by in situ hybridization, it was found that the mRNA existed in the hippocampus, the granular cell layer of cerebellum, cerebral cortex and hypothalamus (Weaver et al., Mol Brain Res 28:296-301, 1995; Weir et al., Proc Natl Acad Sci USA 87:108-112, 1990).

- 35 [0007] Moreover, the distribution of PTH/PTHrP receptor in rat brain matches with the distribution of PTHrP, and so it is assumed that PTHrP acts as a local autocrine/paracrine factor in central nervous system (CNS). An experiment regarding binding of PTH to a cell membrane fraction prepared from each site of rat brain teaches that the order of binding strength is as hypothalamus, cerebellum and cerebral cortex (Harvey et al., Peptides 14:1187-1191, 1993). Furthermore, it has been reported that arginine vasopressin (AVP) is released by PTHrP (1-34) stimulation of rat supraoptic nucleus (SON) slices, and that there is a possibility that PTHrP involves in homeostasis of water or electrolyte in an organism (Yamamoto et al., Endocrinology 139:383-388, 1998; Yamamoto et al., Endocrinology 138:2066-2072, 1997). Thus, although it is clear that PTHrP and a PTH/PTHrP receptor are widely distributed over the brain, physiological role thereof in central nervous system is still unknown.

- 40 [0008] Recently, induction of various types of cytokines by PTHrP or induction of PTHrP by cytokine has been reported, and a new possibility of involvement of PTHrP in various types of diseases caused by cytokine, as well as its own activity, has been clarified. The following reports which suggest the possibility of crosstalk between PTH or PTHrP and cytokine are known:

- 45 1) The values of IL-6 and TNF- α are high in a patient of primary hyperparathyroidism caused by high value of PTH (Grey A. et al., J Clin Endocrinol Metab 81:3450-5, 1996)
50 2) When osteoblasts are stimulated by PTH or PTHrP in an in vitro system, expression of IL-6 and LIF is promoted (Pollock JH. et al., J Bone Miner Res 11:754-9, 1996)
3) A series of experiments with synovial cells showed that production of IL-6 is accentuated by stimulation with PTHrP, and that TNF- α and IL-1 β promote expression of PTHrP, and that therefore PTHrP is a member of pro-inflammatory cytokine cascade (Funk JL. et al., Endocrinology 138:2665-73, 1997; Funk JL. et al., J Clin Invest 101:1362-71, 1998)
55 4) In cultured human vascular endothelial cells also, TNF- α and IL-1 β promote expression of PTHrP (Biochem Biophys Res Commun 249:339-343, 1998)

EP 1 197 225 A1

[0009] Thus, there has been reported induction of cytokine by PTHrP or induction of PTHrP by cytokine etc., and a possibility of involvement of PTHrP in various types of diseases caused by cytokines, especially those such as IL-1 β , IL-6 and TNF- α , as well as its own activity, has been clarified.

[0010] Humoral hypercalcemia of malignancy and cachexia caused by PTHrP and cachexy bring about clinical properties such as body weight loss, weight loss of fat and muscular tissues, anorexia and anemia, and serious damage is given to quality of life (QOL) of a patient. To improve QOL of a cancer patient, application of treatment methods for improving significantly reduced QOL can be considered in addition to normal treatments for the patient with anticancer agents, but at present, there is no treatment method for improving QOL of a patient at a sufficient level. At the moment, treatment methods having recognized QOL improving effect include forced nutrition and pharmacotherapy. With regard to forced nutrition, since the effect is not long-lasting although body weight is maintained, negative opinions have begun to be voiced in the field of palliative care (Tatsuji Kataoka, Blood/Tumor (Ketsueki/Syuryo-ka), 36, 500-506, 1998). With regard to pharmacotherapy, mainly medroxyprogesterone acetate (MPA) is used, but originally MPA is used to treat emmenopathy and breast cancer. MPA shows effects such as stimulation of appetite and fat accumulation, but this is an application of the side effects in treatment of the diseases for which MPA was originally adapted for the purpose of improving QOL. Nevertheless, MPA is not a sufficient therapeutic agent in that this compound is a progestational (gestagenic) formulation, and so it shows original hormone actions, and effects thereof are insufficient.

DISCLOSURE OF THE INVENTION

[0011] The object of the present invention is to provide a therapeutic agent for diseases caused by PTH or PTHrP.

[0012] The present inventors have performed various studies regarding possibility of alleviating symptoms of diseases (treatment for central nervous system diseases, and treatment for diseases caused by PTHrP-cytokine cascade) caused by PTH or PTHrP other than humoral hypercalcemia of malignancy caused by excessive production of PTHrP. As a result, the inventors have found that an anti-PTHrP antibody is effective in alleviating and treating the above symptoms, thereby completing the present invention.

[0013] As shown in Examples described later, it was confirmed that an anti-PTHrP antibody has a high improving effect against symptoms on which no or low improving effects were confirmed using the existing therapeutic agents for hypercalcemia. At the same time, it was also found that the above improvement and treatment effects are not only caused by effects brought by decrease of blood calcium level.

[0014] That is to say, the present invention provides a therapeutic agent for a disease caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor. Mainly, the disease may be one caused by PTH or PTHrP other than hypercalcemia.

[0015] Moreover, the present invention provides a QOL improving agent alleviating symptom of a disease caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.

[0016] Examples of diseases to which the above therapeutic agent and QOL improving agent are applied may include a syndromes associated with malignancy caused by PTHrP (e.g. digestive system disorders such as diarrhea, vomiting and nausea), proteometabolism abnormality (e.g. hypoalbuminemia), saccharometabolism abnormality (e.g. reduction of glucose tolerance and reduction of insulin secretion), lipid metabolism abnormality (e.g. hyperlipidemia and reduction of serum lipoprotein lipase activity), anorexia, hematological abnormality (e.g. anemia, thrombosis and DIC syndrome), electrolyte abnormality (e.g. hyponatremia, hypokalemia and hypercalcemia), immunodeficiency (e.g. infection disease), pain, secondary hyperparathyroidism and primary hyperparathyroidism which are caused by PTH, etc.

[0017] Furthermore, the present invention provides an improving agent for a central nervous system disease caused by PTH or PTHrP which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor. Examples of central nervous system diseases may include dysomnia, neuropathy (e.g. schizophrenia, manic-depressive psychosis, neurosis and psychophysiologic disorder), nervous symptom (e.g. vomiting, nausea, mouth dryness, anorexia and vertigo), brain metabolism abnormality, cerebral circulation abnormality, autonomic imbalance, and endocrine system abnormality with which central nervous system is associated, etc.

[0018] Still further, the present invention provides an improving agent for a disease caused by PTH or PTHrP-cytokine cascade, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor. Examples of cytokines may include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, G-CSF, GM-CSF, M-CSF, EPO, LIF, TPO, EGF, TGF- α , TGF- β , FGF, IGF, HGF, VEGF, NGF, activin, inhibin, a BMP family, TNF and IFN, etc. Examples of diseases caused by PTH or PTHrP-cytokine cascade may include septi-

EP 1 197 225 A1

chemia, cachexia, inflammation, hemopathy such as hematopoietic system abnormality and leukemia, calcium metabolism abnormality, and autoimmune disease such as rheumatism.

[0019] The present invention further provides a central nervous system regulator, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.

[0020] Moreover, the present invention provides a cytokine network regulator, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.

[0021] The PTH receptor or PTHrP receptor may be a PTH/PTHrP type I receptor.

[0022] The substance binding to a ligand of PTH receptor or PTHrP receptor to inhibit binding between the ligand and the receptor may be selected from the group consisting of an anti-PTHrP antibody and an anti-PTH antibody, and among them, an anti-PTHrP antibody, especially a humanized anti-PTHrP antibody is effective.

[0023] The present invention is a therapeutic agent, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.

[0024] In the present specification, the term "a PTH receptor or PTHrP receptor" is used to mean a receptor binding to PTH or PTHrP, and examples include a PTH/PTHrP type I receptor (described in Japanese Patent Application Laying-Open (kohyo) No. 6-506598).

[0025] Examples of "an agonist binding to a PTH receptor or PTHrP receptor" include PTH (1-34), PTH (3-34), PTHrP (1-34), PTHrP (3-34) and an amide form thereof.

[0026] The term "an antagonist binding to a PTH receptor or PTHrP receptor" is used to mean a substance which inhibits binding of PTHrP to a PTH receptor or PTHrP receptor by binding to the PTH receptor or PTHrP receptor (e.g. an antagonist against the PTH receptor or PTHrP receptor (which is also referred to as a PTH or PTHrP antagonist), and specific examples include a PTH or PTHrP peptide comprising a substitution or deletion of at least one amino acid, and a partial sequence of a PTH or PTHrP peptide, etc.). Examples of a PTH or PTHrP antagonist include a polypeptide and a low molecule, and specifically examples of substances antagonistically binding to the receptors against PTH or PTHrP include PTH (7-34), PTH (8-34), PTH (9-34), PTH (10-34), PTHrP (7-34), PTHrP (8-34), PTHrP (9-34), PTHrP (10-34), mutants thereof (e.g. [Nle8, 18, D-Trp12, Tyr34] bovine PTH (7-34) NH₂, [Nle8, 18, Tyr34] bovine PTHrP (7-34)-amide), and an amide form thereof, etc. Examples of substances antagonistically binding to a PTHrP receptor against PTHrP include polypeptides having a PTHrP antagonist activity described in Japanese Patent Application Laying-Open (kokai) No. 7-165790, Japanese Patent Application Laying-Open (kohyo) No. 5-509098, and Peptides (The United States) 1995, 16 (6) 1031-1037, Biochemistry (The United States) Apr. 28, 1992, 31 (16) 4026-4033. Moreover, among the above-stated polypeptides, polypeptides which comprise a deletion, substitution, addition or insertion of at least one amino acid and have an equivalent amount of PTH or PTHrP antagonist activity, are also included in the PTH or PTHrP antagonist of the present invention. However, examples are not limited thereto.

[0027] The term "a ligand" is used to mean a substance binding to an enzyme or receptor.

[0028] The term "a substance binding to a ligand of a PTH receptor or PTHrP receptor to inhibit binding between the ligand and the receptor" is used to mean a substance (e.g. an anti-PTH antibody, an anti-PTHrP antibody, etc.) which inhibits binding of a ligand (e.g. PTH, PTHrP etc.) to a PTH receptor or PTHrP receptor by binding to the ligand of the PTH receptor or PTHrP receptor. Examples of an anti-PTH antibody include an antibody recognizing PTH (1-34) etc. Examples of an anti-PTHrP antibody include antibodies such as a humanized antibody, a human antibody (WO96/33735) and a chimeric antibody (Japanese Patent Application Laying-Open (kokai) 4-228089), and an antibody (a #23-57-137-1 antibody) produced by hybridoma #23-57-137-1, etc. Note that the antibody may be a polyclonal antibody, but a monoclonal antibody is preferable.

[0029] The term "QOL" is an abbreviation for "quality of life" and stands for the quality of life. Cancer patients undergo loss of body weight, anorexia, anemia and pain etc. and so their parameters of QOL are significantly damaged.

[0030] The term "central nervous system" is used to mean a nervous system consisting of brain and spinal cord.

[0031] The term "PTH or PTHrP-cytokine cascade" is described as follows. First, the term "cytokine cascade" stands for a part of cytokine network and means that information of a first cytokine is read by messengers of a second cytokine and a third cytokine. Then, "PTH or PTHrP-cytokine cascade" is used to mean that PTH or PTHrP is included among cytokine members.

[0032] The term "cytokine network" is used to mean antigen-nonspecific network control performed by means of factors (cytokines) produced from immunocompetent cells etc.

[0033] The term "a humanized antibody" is used to mean an antibody comprising frameworks derived from a human antibody and complementarity determining regions (CDRs) derived from an antibody other than human (e.g. a mouse antibody).

[0034] The present specification includes part or all of the contents disclosed in the specification and/or drawings of Japanese Patent Application No. 11-189793, which is a priority document of the present application.

EP 1 197 225 A1

[0035] As an example of the use of "a substance binding to a ligand of a PTH receptor or PTHrP receptor to inhibit binding between the ligand and the receptor", an anti-PTHrP antibody is described below.

1. Anti-PTHrP antibody

[0036] The anti-PTHrP antibody used in the present invention may be any one, regardless of its source, type (monoclonal or polyclonal) and configuration, as long as it can exhibit a desired pharmacological effect.

[0037] The anti-PTHrP antibody used in the present invention can be produced by any known method as a polyclonal or monoclonal antibody. Preferably, the anti-PTHrP antibody is a monoclonal antibody derived from a mammal. The mammal-derived monoclonal antibody includes those produced from a hybridoma and those produced by a genetic engineering technique from a host transformed with a recombinant expression vector carrying a gene for the antibody. The antibody can bind to PTHrP to prevent binding of the PTHrP to a PTH/PTHrP receptor, thus blocking the signal transduction of the PTHrP and consequently inhibiting the biological activity of the PTHrP.

[0038] A specific example of such antibody is #23-57-137-1 antibody which can be produced with a hybridoma clone #23-57-137-1. The hybridoma clone #23-57-137-1 has been designated as "mouse-mouse hybridoma #23-57-137-1" and deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) under the accession No. FERM BP-5631.

2. Antibody-producing hybridoma

[0039] A monoclonal antibody-producing hybridoma can be produced as follows. That is, PTHrP is used as an antigen for immunization in accordance with a conventional immunization method. The resulting immunocytes are fused to known parent cells by a conventional cell fusion method, and monoclonal antibody-producing cells are screened from the fused cells by a conventional screening method.

[0040] First, a human PTHrP, which is used as a sensitizing antigen for producing the antibody, is prepared by expressing the PTHrP gene/amino acid sequence disclosed in Suva, L. J. et al., *Science* (1987) 237, 693. A nucleotide sequence encoding the PTHrP is inserted into a known expression vector, and a suitable host cell is transformed with the expression vector. The PTHrP protein is then isolated and purified from the transformed host cell or from a culture supernatant of the transformed host cell by any known method.

[0041] Then, the purified PTHrP protein is used as a sensitizing antigen. Alternatively, a 34-amino acid peptide of the N-terminal region of the PTHrP may be chemically synthesized as the sensitizing antigen.

[0042] The mammal to be immunized with the sensitizing antigen is not particularly limited. However, the mammal is preferably selected taking into consideration of compatibility with the patent cell used for cell fusion. Generally, a rodent (e.g., mouse, rat, hamster), rabbit or monkey may be used.

[0043] The immunization of the mammal with the sensitizing antigen can be performed in accordance with any known method, for example, by injecting the sensitizing antigen to a mammal intraperitoneally or subcutaneously. More specifically, the sensitizing antigen is properly diluted with or suspended to phosphate-buffered saline (PBS) or physiological saline, the resulting dilution or suspension is then mixed with an appropriate amount of a conventional adjuvant (e.g., Freund's complete adjuvant) to give an emulsion. The emulsion is injected to a mammal several times at intervals of 4 to 21 days. For the immunization, the sensitizing antigen may be attached to a suitable carrier.

[0044] After the immunization, the serum antibody level is checked. When the serum antibody level is confirmed to reach a desired level, immunocytes are isolated from the mammal and then subjected to cell fusion. A preferable immunocyte is a spleen cell.

[0045] The parent cell used for the cell fusion (i.e., the counterpart of the cell fusion with the immunocyte) is a myeloma cell derived from a mammal. The myeloma cell is of any known cell line, and, for example, P3 (P3x63Ag8.653) (J. Immunol. (1979) 123, 1548-1550), P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D. H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), FO (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148, 313-323) or R210 (Galfre, G. et al., Nature (1979) 277, 131-133).

[0046] Cell fusion of the immunocyte to the myeloma cell is basically performed in accordance with any known method, such as the method of Milstein et al. (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

[0047] More specifically, the cell fusion is performed, for example, in a conventional nutrient culture medium in the presence of a cell fusion promoter. The cell fusion promoter may be polyethylene glycol (PEG) or a Sendai virus (hemagglutinating virus of Japan; HJV). If desired, for the purpose of improving the fusion efficiency, an additive such as dimethyl sulfoxide may be incorporated.

[0048] The ratio between the immunocytes and the myeloma cells for the cell fusion may be any one. For example,

EP 1 197 225 A1

the immunocytes are used in the amount 1-10 times larger than the myeloma cells. The culture medium used for the cell fusion is, for example, RPMI 1640 medium or MEM medium suitable for the growth of the above-mentioned myeloma cell lines, or other medium conventionally used for the culture of such cell lines. If desired, a serum supplement, such as fetal calf serum (FCS), may be added to the culture medium.

5 [0049] The cell fusion is performed by fully mixing given amounts of the immunocytes and the myeloma cells in the culture medium, adding a PEG solution (e.g., mean molecular weight: about 1000-6000) (which has been previously warmed to about 37° C) to the mixture usually to a concentration of 30-60% (w/v), and then mixing the resulting solution, thereby producing the desired fusion cells (i.e., hybridomas). Subsequently, an appropriate culture medium is added to the culture solution successively, and centrifuged to remove the supernatant. This procedure is repeated several times to remove the cell fusion promoter or the like that are undesirable for the growth of the hybridomas, from the culture medium.

10 [0050] The thus obtained hybridomas can be selected by culturing in a conventional selective medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium. The culturing of the hybridomas in HAT medium is performed for the time of period enough to cause the death of the cells other than the desired hybridomas (i.e., cells that fail to fuse), usually for several days to several weeks. Subsequently, conventional limiting dilution method is performed for screening and mono-cloning of the hybridomas that are secreting the desired antibody.

15 [0051] As a method other than preparing the hybridomas by immunizing a non-human mammal with the antigen as described above, a human lymphocyte may be sensitized with PTHrP in vitro, and then subjected the sensitized lymphocyte to cell fusion to a human-derived myeloma cell capable of infinite growth, thereby producing a human antibody having a binding activity against the PTHrP (Japanese Patent Publication No. 1-59878). Alternatively, a human antibody against PTHrP may be prepared by injecting PTHrP as an antigen to a transgenic animal that has the entire repertoires of human antibody genes to produce an anti-PTHrP antibody-producing cell, and then immortalizing the cells, thus producing the human antibody from the immortalized cell (International Patent Publication Nos. WO 94/25585, WO 93/12227, WO 92/03918 and WO 94/02602).

20 [0052] The monoclonal antibody-producing hybridoma prepared as above can be subcultured in a conventional culture medium and stored under liquid nitrogen for a long time of period.

25 [0053] For the production of a monoclonal antibody from the hybridoma, a method may be employed that involves culturing the hybridoma in accordance with a conventional technique and collecting the monoclonal antibody from the culture supernatant, or that involves injecting the hybridoma to a mammal compatible with the hybridoma to grow the hybridoma in the mammal and collecting the hybridoma from the ascites of the mammal. The former method is suitable for producing the antibody in high purity, while the latter method is suitable for producing the antibody in a large amount.

3. Recombinant antibody

35 [0054] In the present invention, a recombinant-type monoclonal antibody may be used, which can be produced by cloning an antibody gene from the hybridoma, integrating the antibody gene into a suitable vector, introducing the vector into a host, and then producing the antibody from the host according to a conventional genetic recombination technique (see, for example, Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192, 767-775, 1990)

40 [0055] Specifically, mRNA encoding variable (V) region of an anti-PTHrP antibody is isolated from the anti-PTHrP antibody-producing hybridoma. The isolation of the mRNA is performed by preparing a total RNA by any known method, such as guanidium ultracentrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) and AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159), and then producing the desired mRNA from the total RNA using mRNA Purification Kit (Pharmacia) or the like. Alternatively, the mRNA may also be prepared directly using QuickPrep mRNA Purification Kit (Pharmacia).

45 [0056] Next, cDNA for the antibody V-region is synthesized from the mRNA with a reverse transcriptase. The synthesis of the cDNA is performed using AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation) or the like. The cDNA may also be synthesized and amplified by 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) using 5'-Amplicon FINDER RACE Kit (CLONETECH) in combination with PCR method, or the like.

50 [0057] A DNA fragment of interest is isolated and purified from the resulting PCR product and then ligated to a vector DNA to obtain a recombinant vector. The recombinant vector is introduced into a host such as E. coli, and a colony containing a desired recombinant vector is selected. The nucleotide sequence of the DNA of interest in the recombinant vector is confirmed by, for example, dideoxynucleotide chain termination method.

55 [0058] Once DNA encoding the anti-PTHrP antibody V-region is obtained, the DNA is integrated into an expression vector containing a DNA encoding a desired antibody constant (C) region.

[0059] For the production of the anti-PTHrP antibody used in the present invention, the antibody gene is integrated into an expression vector so that the antibody gene can be expressed under the control of expression control regions (e.g., enhancer, promoter). A host cell is transformed with the expression vector to express the antibody.

EP 1 197 225 A1

[0060] In the expression of the antibody gene, a DNA encoding heavy (H) chain and a DNA encoding light (L) chain of the antibody may be integrated into separate expression vectors, and then a host cell is co-transformed with the resulting recombinant expression vectors. Alternatively, both the DNA encoding H-chain and the DNA encoding L-chain of the antibody may be integrated together into a single expression vector, and then a host cell may be transformed with the resulting recombinant expression vector (WO 94/11523).

[0061] For the production of the recombinant antibody, besides the above-mentioned host cells, a transgenic animal may also be used as a host. For example, the antibody gene is inserted into a predetermined site of a gene encoding a protein inherently produced in the milk of an animal (e.g., goat β -casein) to obtain a fusion gene. A DNA fragment containing the antibody gene-introduced fusion gene is injected into an embryo of a goat, and the embryo is then introduced into a female goat. The female goat having the embryo therein bears a transgenic goat. The antibody of interest is secreted in the milk from the transgenic goat or a progeny thereof. For the purpose of increasing the amount of the antibody-containing milk from the transgenic goat, an appropriate hormone may be administered to the transgenic goat (Ebert, K.M. et al., *Bio/Technology* (1994) 12, 699-702).

4. Modified antibody

[0062] In the present invention, for the purpose of reducing the heterogeneity against a human body or the like, an artificially modified recombinant antibody may be used, such as a chimeric antibody and a humanized antibody. These modified antibodies can be prepared by the following known methods.

[0063] A chimeric antibody usable in the present invention can be prepared by ligating the DNA encoding the antibody V-region prepared as set forth above to a DNA encoding a human antibody C-region, integrating the ligation product into an expression vector, and introducing the resulting recombinant expression vector into a host to produce the chimeric antibody.

[0064] A humanized antibody is also referred to as a "reshaped human antibody", in which the complementarity determining regions (CDRs) of an antibody of a non-human mammal (e.g., a mouse) are grafted to those of a human antibody. The general genetic recombination procedures for producing such humanized antibody are also known (EP 125023; WO 96/02576).

[0065] Specifically, a DNA sequence in which mouse antibody CDRs are ligated through framework regions (FRs) of a human antibody is amplified by PCR method using several oligonucleotides as primers which have been designed to have regions overlapping to the terminal regions of the CDRs and the FRs. The resulting DNA is ligated to a DNA encoding a human antibody C-region, and the ligation product is integrated into an expression vector. The resulting recombinant expression vector is introduced into a host, thereby producing the humanized antibody (EP 239044, WO 96/02576).

[0066] The FRs of the human antibody ligated through the CDRs are selected so that the CDRs can form a suitable antigen binding site. If necessary, an amino acid(s) in the FRs of the antibody V-region may be replaced so that the CDRs of the reshaped human antibody can form a suitable antigen binding site (Sato, K. et al., *Cancer Res.* (1993) 53, 851-856).

[0067] The C-region of the chimeric or humanized antibody may be any human antibody C-region, such as C γ 1, C γ 2, C γ 3 or C γ 4 for the H-chain, and C κ or C λ for the L-chain. The human antibody C-region may be modified for the purpose of improving the stable production of the antibody.

[0068] The chimeric antibody is composed of V-regions derived from a non-human mammalian antibody and C-regions derived from a human antibody. The humanized antibody is composed of CDRs derived from a non-human mammalian antibody and FRs and C-regions derived from a human antibody. The humanized antibody is useful as an active ingredient for the agent of the present invention, because the antigenicity of the antibody against a human body is reduced.

[0069] A specific example of the humanized antibody usable in the present invention is humanized #23-57-137-1 antibody; in which the CDRs are derived from mouse-derived #23-57-137-1 antibody; the L-chain is composed of the CDRs ligated through three FRs (FR1, FR2 and FR3) derived from human antibody HSU 03868 (GEN-BANK, Dertos, M. et al., *Scand. J. Immunol.*, 39, 95-103, 1994) and a FR (FR4) derived from human antibody S25755 (NBRF-PDB); and the H-chain is composed of the CDRs ligated through FRs derived from human antibody S31679 (NBRF-PDB, Cuisinier, A. M. et al., *Eur. J. Immunol.*, 23, 110-118, 1993) in which a part of the amino acid residues in the FRs is replaced so that the reshaped humanized antibody can exhibit an antigen-binding activity.

[0070] The E. coli strains containing plasmids having DNA encoding the H-chain and the L-chain of the humanized #23-57-137-1 antibody are designated as *Escherichia coli* JM109 (hMBC1HcDNA/pUC19) (for H-chain) and *Escherichia coli* JM109 (hMBC1Lq λ /pUC19) (for L-chain), respectively. These strains were deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan), under the accession No. FERM BP-5629 for *Escherichia coli* JM109 (hMBC1HcDNA/pUC19), and under the accession No. FERM BP-5630

EP 1 197 225 A1

for *Escherichia coli* JM109 (hMCB1Lq λ pUC19).

5. Antibody variants

- [0071]** The antibody used in the present invention may be a fragment thereof or a modified form of the fragment, as long as it binds to PTHrP to inhibit the activity thereof. For example, the fragment of the antibody includes Fab, F(ab')₂, Fv, or a single chain Fv (scFv) composed of a H-chain Fv fragment and a L-chain Fv fragment linked together through a suitable linker. Specifically, such antibody fragments can be produced by cleaving the antibody with an enzyme (e. g., papain, pepsin) into antibody fragments, or by constructing a gene encoding the antibody fragment and inserting the gene into an expression vector and introducing the resulting recombinant expression vector into a suitable host cell, thereby expressing the antibody fragment (see, for example, Co, M. S., et al., *J. Immunol.* (1994), 152, 2968-2976; Better, M. & Horwitz, A. H., *Methods in Enzymology* (1989), 178, 476-496, Academic Press, Inc.; Plueckthun, A. & Skerra, A., *Methods in Enzymology* (1989) 178, 476-496, Academic Press, Inc.; Lamoyi, E., *Methods in Enzymology* (1989) 121, 652-663; Rousseaux, J. et al., *Methods in Enzymology* (1989) 121, 663-669; and Bird, R. E. et al., *TIBTECH* (1991) 9, 132-137).
- [0072]** A scFv can be produced by linking the H-chain V-region to the L-chain V-region through a linker, preferably a peptide linker (Huston, J. S. et al., *Proc. Natl. Acad. Sci. USA* (1988) 85, 5879-5883). The H-chain V-region and the L-chain V-region in the scFv may be derived from any one of the antibodies described herein. The peptide linker which binds the V-regions may be any single chain peptide, for example, of 12-19 amino acid residues.
- [0073]** The DNA encoding the scFv can be prepared by first amplifying a DNA encoding the H-chain V-region and a DNA encoding the L-chain V-region of the antibody separately using a DNA fragment encoding the entire region or a part of the H-chain that includes the V-region and a DNA fragment encoding the entire region or a part of the L-chain that includes the V-region as templates and primer pairs that define the terminal ends of the DNA fragments; and then amplifying a DNA encoding the peptide linker using a DNA fragment encoding the peptide linker as a template and a primer pair that define the terminal ends of the DNA fragment so that each terminal end of the peptide linker is ligated to the H-chain V-region and the L-chain V-region, respectively.
- [0074]** Once the DNA encoding the scFv is prepared, an expression vector carrying the DNA and a host transformed with the expression vector can be prepared by conventional methods. The scFv can be produced from the transformed host by a conventional method.
- [0075]** The fragments of the antibody may be produced by preparing genes for the fragments and expressing the genes in suitable hosts as described above. The antibody fragments is also encompassed in the "antibody" of the present invention.
- [0076]** As a modified form of the above-mentioned antibodies, for example, anti-PTHrP antibody conjugated to any molecule (e.g., polyethylene glycol) may also be used. Such modified antibodies are also encompassed in the "antibody" of the present invention. The modified antibodies can be prepared by chemical modifications of the antibodies. The chemical modification techniques suitable for this purpose have already been established in the art.

6. Expression and production of recombinant antibody or modified antibody

- [0077]** The antibody gene constructed as described above can be produced and expressed by known methods. For the expression in a mammalian cell, a conventional useful promoter, the antibody gene to be expressed and a poly(A) signal (located downstream to the 3' end of the antibody gene) are operably linked. For example, as the useful promoter/enhancer system, a human cytomegalovirus immediate early promoter/enhancer system may be used.
- [0078]** Other promoter/enhancer systems usable in the expression of the antibody used in the present invention include those derived from viruses (e.g., retrovirus, polyoma virus, adenovirus and simian virus 40 (SV40)) and those derived from mammalian cells (e.g., human elongation factor 1 α (HEF1 α)).
- [0079]** When SV40 promoter/enhancer system is used, the gene expression may be performed readily by the method of Mulligan et al. (*Nature* (1979) 277, 108). When HEF1 α promoter/enhancer system is used, the gene expression may be performed readily by the method of Mizushima et al. (*Nucleic Acids Res.* (1990) 18, 5322).
- [0080]** For the expression in *E. coli*, a conventional useful promoter, a signal sequence for secreting the antibody of interest and the antibody gene may be operably linked. As such a promoter, lacZ promoter or araB promoter may be used. When lacZ promoter is used, the gene expression may be performed by the method of Ward et al. (*Nature* (1988) 341, 544-546; FASEB J. (1992) 6, 2422-2427). When araB promoter is used, the gene expression may be performed by the method of Better et al. (*Better et al., Science* (1988) 240, 1041-1043).
- [0081]** Regarding the signal sequence for secretion of the antibody, when the antibody of interest is intended to be secreted in a periplasmic space of the *E. coli*, pelB signal sequence (Lei, S. P. et al., *J. Bacteriol.* (1987) 169, 4379) may be used. The antibody secreted into the periplasmic space is isolated and then refolded so that the antibody takes an appropriate configuration for use.

EP 1 197 225 A1

[0082] Regarding the replication origin, those derived from viruses (e.g., SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV)) or the like may be used. In order to increase the gene copy number in the host cell system, the expression vector may further contain a selective marker gene, such as an aminoglycoside phosphotransferase (APH) gene, a thymidine kinase (TK) gene, an *E. coli* xanthine-guanine phosphoribosyltransferase (Ecogpt) gene and a dihydrofolate reductase (dhfr) gene.

[0083] For the production of the antibody used in the present invention, any expression system such as eukaryotic and prokaryotic cell systems may be used. The eukaryotic cell includes established cell lines of animals (e.g., mammals, insects, molds and fungi, yeast). The prokaryotic cell includes bacterial cells such as *E. coli* cells. It is preferable that the antibody used in the present invention be expressed in a mammalian cell, such as a CHO, COS, myeloma, BHK, Vero or HeLa cell.

[0084] Next, the transformed host cell is cultured *in vitro* or *in vivo* to produce the antibody of interest. The culturing of the host cell may be performed by any known method. The culture medium usable herein may be DMEM, MEM, RPMI 1640 or IMDM medium. The culture medium may contain a serum supplement, such as fetal cell serum (FCS).

7. Isolation and purification of antibody

[0085] The antibody expressed and produced as described above may be isolated from the cells or the host animal body and purified to uniformity. The isolation and purification of the antibody used in the present invention may be performed on an affinity column. Examples of a protein A column include Hyper D, POROS and Sepharose F.F. (Pharmacia). The method is not particularly limited and other methods conventionally used for the isolation and purification of an antibody may also be employed. For example, various chromatographs using columns other than the above-mentioned affinity column, filtration, ultrafiltration, salting out and dialysis may be used singly or in combination to isolate and purify the antibody of interest (Antibodies A Laboratory Manual, Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

8. Determination of the activities of the antibody

[0086] The determination of the antigen-binding activity (Antibodies A Laboratory Manual, Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988) or the inhibitory activity against a ligand receptor (Harada, A. et al., International Immunology (1993) 5, 681-690) of the antibody used in the present invention may be performed by any known methods.

[0087] The method for the determination of the antigen-binding activity of the anti-PTHrP antibody used in the present invention may be ELISA (enzyme-linked immunosorbent assay), EIA (enzyme immunoassay), RIA (radioimmunoassay) or a fluorescent antibody. For example, when enzyme immunoassay is employed, a sample solution containing the anti-PTHrP antibody (e.g., a culture supernatant of anti-PTHrP antibody-producing cells, or the anti-PTHrP antibody in a purified form) is added to a plate on which PTHrP (1-34) is previously coated. A secondary antibody labeled with an enzyme (e.g., alkaline phosphatase) is further added to the plate. The plate is incubated and washed. A substrate for the enzyme (e.g., p-nitrophenylphosphoric acid) is added to the plate, and the absorbance of the solution in the plate is measured to evaluate the antigen-binding activity of the antibody.

[0088] To confirm the activity of the antibody used in the present invention, a neutralizing activity of the antibody (e.g., anti-PTHrP antibody) may be determined.

9. Routes for administration and pharmaceutical preparations

[0089] The agent of the present invention can be used as a therapeutic agent for diseases caused by PTH or PTHrP; a QOL improving agent alleviating symptoms of diseases caused by PTH or PTHrP; an improving agent for central nervous system diseases caused by PTH or PTHrP; an improving agent for diseases caused by PTH or PTHrP-cytokine cascade; a central nervous system regulator; and a cytokine network regulator etc. The agent of the present invention can be administered for any one or a plurality of the above uses.

[0090] An agent containing the anti-PTHrP antibody of the present invention as an active ingredient may be administered orally or parenterally, but preferably parenterally. Specifically, the agent may be administered to a body as a whole or regionally, taking any dosage form, such as a transpulmonary agent (e.g., an agent administered with the help of a device such as a nebulizer), a nasogastric agent, a transdermic agent (e.g., ointment, cream) and an injection. Examples of an injection include an intravenous injection such as a drip, an intramuscular injection, an intraperitoneal injection and a subcutaneous injection. The route of administration may be properly selected depending on the age of a patient and the conditions of diseases. An effective single dose may be selected within the range from 0.001 to 1,000 mg per kg of body weight. Alternatively, the dose to a patient may be selected within the range from 0.01 to 100,000 mg/body, preferably 0.1 to 10,000 mg/body, more preferably 0.5 to 1,000 mg/body, and further more preferably 1 to 100mg/body. However, the dose of the agent comprising the anti-PTHrP antibody of the present invention is not par-

EP 1 197 225 A1

ticularly limited to these ranges.

[0091] The agent may be administered to a patient at any stage, including before or after the development of disease or symptoms, or may also be administered at a stage when body weight reduction is predicted in a patient.

[0092] The agent comprising the anti-PTHrP antibody as an active ingredient of the present invention may be formulated by any conventional method (Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, USA). The preparation may further comprise pharmaceutically acceptable carriers and additives.

[0093] Examples of such carriers and additives include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinyl pyrrolidone, carboxyvinyl polymer, sodium carboxymethyl cellulose, poly(sodium acrylate), sodium arginate, water soluble dextran, sodium carboxymethyl starch, pectin, methyl cellulose, ethyl cellulose, xanthane gum, gum arabic, casein, agar, polyethylene glycol, diglycerin, glycerin, propylene glycol, vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, and surfactants acceptable as pharmaceutical additives.

[0094] In the practical use, the additive is properly selected from the above members either singly or in combination depending on (without limitation) the dosage form of the agent of the present invention employed. For example, for use as an injectable form, the anti-PTHrP antibody of the purified form is dissolved in a solvent (e.g., physiological saline, a buffer, a grape sugar solution) and then an adsorption-preventing agent (e.g., Tween 80, Tween 20, a gelatin, human serum albumin) is added thereto. The therapeutic agent of the present invention may also be in a re-constitutable freeze-dried form, which is dissolved before use. For the formulation of the freeze-dried dosage form, an excipient such as a sugar alcohol (e.g., mannitol, grape sugar) or a sugar may be incorporated.

BRIEF DESCRIPTION OF DRAWINGS

[0095]

Fig. 1 is a figure showing effect of a humanized anti-PTHrP antibody on blood vasopressin level in high PTHrP-related hypercalcemia model rats.

Fig. 2 is a figure showing effect of a humanized anti-PTHrP antibody on urine volume in high PTHrP-related hypercalcemia model rats.

Fig. 3 is a figure showing life-prolonging effect of a humanized anti-PTHrP antibody in septicemia model rats.

Fig. 4 is a figure showing the results of pharmacological study of a humanized anti-PTHrP antibody and alendronate in high PTHrP-related hypercalcemia model rats.

Fig. 5 is a figure showing effect of a humanized anti-PTHrP antibody on amount of autonomic movement in high PTHrP-related hypercalcemia model rats.

BEST MODE FOR CARRYING OUT THE INVENTION

[0096] The present invention is further described in the following examples. However, the examples are provided for illustrative purposes only, and are not intended to limit the scope of the invention.

[EXAMPLE 1]

Low vasopressin level improving effect by anti-PTHrP antibody

[0097] A living organism has control mechanisms for maintaining various electrolytes in body fluid at their optimum levels in order to control its body water content. Vasopressin (alias: antidiuretic hormone (ADH), one kind of posterior pituitary hormones) is known as a hormone controlling metabolism of electrolyte and water. Further, various types of diseases caused by disorder of this hormone are also known. Examples of such diseases include posterior pituitary gland hyperplasia (diabetes insipidus) and vasopressin secretion abnormality etc.

[0098] Among these symptoms, polydipsia, polyuria and mouth dryness etc. are particularly characteristic clinical symptoms. PTHrP is known as a causal substance for humoral hypercalcemia of malignancy (HHM). The onset mechanism of HHM, which is caused by PTHrP produced by malignancy, relates to promotion of bone resorption and promotion of calcium resorption in the kidneys. It is known that, once hypercalcemia develops, polyuria caused by vasopressin adipsia or dehydration relative to anorexia, vomiting and nausea caused by the disease itself occurs, and thereby hypercalcemia being more promoted.

EP 1 197 225 A1

(1) Purpose:

[0099] Using a human high PTHrP-related hypercalcemia model animal, human tumor inoculated nude rat model, the effect of a humanized anti-PTHrP monoclonal antibody on blood vasopressin level and urine volume in the model animal was examined.

(2) Methods:

[0100] As a model animal, a nude rat implanted with human large cell lung carcinoma LC-6 [purchased from the Central Institute for Experimental Animals] was used. It is known that a nude rat implanted with human large cell lung carcinoma LC-6 shows increased blood calcium level along with production of PTHrP as tumor volume increases, and develops loss of body weight and so on. The blood vasopressin level in this human high PTHrP-related hypercalcemia model animal was measured, and the value was compared with that of a normal rat. Further, the effect of a humanized anti-PTHrP monoclonal antibody on blood vasopressin level was analyzed. Furthermore, urine volume was measured and the effect of a humanized anti-PTHrP monoclonal antibody on urine volume was also analyzed.

[0101] Preparation and grouping of human high PTHrP-related hypercalcemia model animals were carried out as follows. Using a BALB/c-nu/nu nude mouse (CLEA Japan, Inc.), in vivo transplanted human large cell lung carcinoma LC-6 was removed from the nude mouse, and then finely cut into 3-mm cubic blocks. The resulting tumor blocks were subcutaneously implanted into each of the rats at the lateral region at a ratio of one piece per mouse. As rats, 5-week-old male F344/N Jcl-mu nude rats (CLEA Japan, Inc.) were purchased and acclimatized for 1 week. The resulting 6-week-old rats were implanted with the tumor blocks, and about a month and a half after the implantation, the rats with increased blood calcium levels and reduced body weights were used as human high PTHrP-related hypercalcemia model animals for evaluation of drug efficacy. Using blood calcium level and body weight as indicators, the rats were divided into groups so that blood calcium levels and body weights of the rats in the individual groups were averaged.

[0102] In experiments regarding vasopressin level measurement, 3mg/kg humanized anti-PTHrP monoclonal antibody were administered to human high PTHrP-related hypercalcemia model animals prepared and divided into groups by the above method via caudal vein once a week, that is, on the day 0, 7, 14, 21, 28 and 35. Alendronate was administered to the model animals via caudal vein twice a week, that is, on the day 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35 and 38 at a dose level of 2.5 mg/kg. As a control, phosphate buffered saline (PBS) was administered to each of another group of the model animals via caudal vein on the day 0, 7, 14, 21, 28 and 35. Where an implanted tumor block was clearly decalcified during the experiment period, it was eliminated from counting of results.

[0103] For the measurement of blood vasopressin level, blood plasma was collected from descending aorta and separated with EDTA. Since data of individuals whose implanted tumor was clearly decalcified when the blood was collected was eliminated from counting, the number of each group at the time of blood collection was: 12 rats in a humanized anti-PTHrP monoclonal antibody administration group, 3 rats in an alendronate administration group, 8 rats in phosphate buffered saline (PBS) administration group and 5 rats in a normal rat group. The measurement was carried out by RIA method using blood plasma.

[0104] In experiments regarding urine volume measurement, 3mg/kg humanized anti-PTHrP monoclonal antibody or 5mg/kg alendronate were administered to human high PTHrP-related hypercalcemia model animals prepared and divided into groups by the above method via caudal vein. As a control, phosphate buffered saline (PBS) was administered to the model animals via caudal vein. On the morning of the day 13 to the morning of the day 14 after administration, urine was collected over 24 hours, and then weight and specific gravity were measured to calculate urine volume.

(3) Results:

[0105] It was found that blood vasopressin level was reduced in human high PTHrP-related hypercalcemia model animals. A humanized monoclonal antibody improved the reduced blood vasopressin level in human high PTHrP-related hypercalcemia model animals (Figure 1). Furthermore, it was also found that the humanized monoclonal antibody has an effect of improving the state of polyuria in human high PTHrP-related hypercalcemia model animals (Figure 2). From these results, it turned out that a humanized anti-PTHrP monoclonal antibody effects recovery from dehydration by means of normalization of blood vasopressin level.

[EXAMPLE 2] Life-prolonging effect of anti-PTHrP antibody in septicemia model animals Possibility of humanized anti-PTHrP monoclonal antibody as therapeutic agent for septicemia (a therapeutic agent for septicemia)

[0106] Septicemia (sepsis) is state wherein microorganisms such as bacteria and fungus, and metabolites thereof continuously spread from an in vivo infectious focus via the circulating blood. Clinical symptoms of this disease are

EP 1 197 225 A1

fever, ague, shaking chill, tachycardia and consciousness disorder etc., and after the progression of the disease, it results in septic shock and combines with disorders of various organs such as circulatory failure, disseminated intravascular coagulation syndrome (DIC), adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF) etc., and this disease still shows a high mortality rate.

[0107] Triggered by endotoxin derived from bacteria, these various clinical symptoms are developed by progression of a series of reactions, what is known as septicemia cascade. It is known that, above all, the fatty portion of endotoxin constituting cell wall of Gram-negative bacteria (LPS: lipopolysaccharide) has a strong physiological action. In recent years, septicemia has been clarified at the cytokine level, and it has been found that, in septic shock caused by LPS, cytokines such as IL-1, IL-6, IL-8 and IFN γ increase in serum.

[0108] Moreover, Funk et al. has reported that, when a large amount of LPS is administered to a mouse, a parathyroid hormone-related peptide (PTHrP) is produced and induced, while various types of cytokines are produced and induced. Still more, it has been reported that life-prolonging effect can be obtained when a goat anti-PTHrP antibody and a rabbit anti-PTHrP antibody are administered to this model (Mol Med 2, 204, 1996).

[0109] Establishing life-prolonging effect as an indicator, a retest was performed to examine whether the same effect can be obtained by a humanized anti-PTHrP monoclonal antibody, and the adaptability of the humanized anti-PTHrP monoclonal antibody as a therapeutic agent for septicemia.

Effect of humanized anti-PTHrP monoclonal antibody against septicemia model animals

(1) Purpose:

[0110] Effect of a humanized anti-PTHrP monoclonal antibody on model animals with septicemia induced by LPS was analyzed to expand adaptability of this antibody.

(2) Methods:

[0111] According to the method of Funk et al., this experiment was carried out.

1. As model animals, 6-week-old normal Jcl:ICR mice (CLEA Japan, Inc.) which were acclimatized for 1 week were used, and septicemia-developed mice were prepared by administration of LPS. Each of 700, 800 and 900 $\mu\text{g}/\text{mouse}$ LPS (E. coli 055 : B5 (Difco)) was intraperitoneally administered to mice ($n=3$), and conditions until death were observed over time. 800 $\mu\text{g}/\text{mouse}$ LPS was selected as a dose with which 80% or more mice die over 48 hours.

2. Evaluation of drug efficacy of a humanized anti-PTHrP monoclonal antibody (an antibody comprising, as an L-chain, version q described later) was performed by the following method. Concurrently with (concurrent administration group : $n=12$) or at 1 hour after (prior administration group : $n=13$) the administration of 1,000 $\mu\text{g}/\text{mouse}$ humanized anti-PTHrP monoclonal antibody to mice via caudal vein, 800 $\mu\text{g}/\text{mouse}$ LPS was intraperitoneally administered thereto ($n=13$). The succeeding conditions to death were observed with time (0 to 72 hours), and life-prolonging effect in a humanized anti-PTHrP monoclonal antibody was analyzed by making a comparison between a group to which the antibody was administered and a control group.

(3) Results:

[0112] A large number of mice died at 36 hours after administration of LPS in all groups. Survival rate after 48 hours was 15.4% in a control group, 33.3% in a concurrent administration group and 30.8% in a prior administration group, then after 72 hours, 7.7% in a control group, 25.0% in a concurrent administration group and 30.8% in a prior administration group. This result shows that the concurrent or prior administration of a humanized anti-PTHrP monoclonal antibody brought about a life-prolonging effect in LPS-induced septicemia model animals (Figure 3). Accordingly, it was suggested that a humanized anti-PTHrP monoclonal antibody is useful as a therapeutic agent for septicemia.

[EXAMPLE 3]

QOL improving effect of anti-PTHrP antibody in human high PTHrP-related hypercalcemia model animals

[0113] The present inventors had already found that a humanized anti-PTHrP antibody shows not only reduction of blood calcium level but also significant rebound of body weight, increase of amount of autonomic movement and increase of food consumption in human high PTHrP-related hypercalcemia model animals (Japanese Patent Application

EP 1 197 225 A1

Laying-Open (kokai) No. 11-92500). There remains some doubt whether the improving effect of QOL parameters such as body weight, amount of autonomic movement and food consumption is provided by improving or corrected to a normal value, blood calcium level, or whether it depends on factors other than stated above. Thus, the present inventors have analyzed the relation between reduction of blood calcium level and increase of body weight, amount of autonomic movement, and food and water consumption. As a brief explanation of the experiment, high PTHrP-related hypercalcemia model animals were prepared by subcutaneously implanting a PTHrP-producing tumor, human large cell lung carcinoma LC-6, to rats. After that, 3mg/kg humanized anti-PTHrP monoclonal antibody were intravenously administered to the thus prepared model animals. As a control group, 5mg/kg alendronate (a bisphosphonate formulation) were intravenously administered (a single administration). Then, blood ionized calcium (iCa) level, body weight, food and water consumption of the animals were measured. From results of parameters obtained from this experiment, an accumulated value (an area calculated from a graph, setting the stalling point of agent administration starting point as 0 level). Regarding body weight, however, the value was directly used. The graph was prepared, setting the accumulated value of blood calcium level reduction at a horizontal axis and the accumulated value of various parameters at a vertical axis. 5mg/kg alendronate were administered as a control agent. The results are shown below. It was found that, in all of the parameters in comparison with reduction of blood calcium level, the gradient of a line obtained regarding a humanized anti-PTHrP antibody differs from that obtained regarding alendronate (Figure 4A, B, C and D). Especially regarding amount of autonomic movement (Figure 4B) and water consumption (Figure 4D), the sign (positive and negative) of the line was reversed. This result suggests that, in respect of reduction of blood calcium level, a humanized anti-PTHrP antibody provides a much higher QOL improving effect than alendronate (a bisphosphonate formulation). Accordingly, it was suggested that PTHrP is not only a causal substance of humoral hypercalcemia of malignancy, but also a cause of malignancy-associated syndrome by means of a mechanism other than calcium.

[0114] Moreover, it became clear that an anti-PTHrP antibody has a significantly higher QOL improving effect than the existing therapeutic agents for hypercalcemia.

[EXAMPLE 4]

(1) Purpose:

[0115] The effect of a humanized anti-PTHrP monoclonal antibody on autonomic movement of high PTHrP-related hypercalcemia model animals was examined.

(2) Methods:

[0116] High PTHrP-related hypercalcemia model animals were prepared by subcutaneously implanting a PTHrP-producing tumor, human large cell lung carcinoma LC-6, to nude rats. After that, PBS (control) or 5mg/kg humanized anti-PTHrP monoclonal antibody were intravenously administered to the thus prepared model animals (a single administration). Amount of autonomic movement of rats was measured by placing each rat in an individual cage and using an amount of autonomic movement counting device, ANIMEX, counting the frequency. A count was performed on the day 1, 3 and 5 after administration for PBS-administered individuals, and on the day 0 (a pre-administration value), 2, 4 and 6 days for antibody-administered individuals. The count period was for 12 hours, from 7:00PM to 7:00AM the following morning.

(3) Results:

[0117] Results were obtained by counting every hour. It is known that autonomic movement patterns of a normal rat has a periodic rhythm. It was found that periodicity of autonomic movement can be observed, but the pattern is irregular in the present high PTHrP-related hypercalcemia models (from results of PBS-administered individuals). In contrast, in antibody-administered individuals, it was found that periodic pattern of the autonomic movement becomes notable in conjunction with increase of the autonomic movement (Figure 5).

(4) Consideration:

[0118] Autonomic movement, in particular, periodicity of the movement etc. is controlled by motor nerve etc. in the central nerve system. In high PTHrP-related hypercalcemia models subjected to the present experiment, not only reduction of amount of movement but also disorder of the periodicity was observed, and effect of high PTHrP-related hypercalcemia on the central nerve system was assumed. From the result that the periodicity of autonomic movement became notable as a result of administration of a humanized monoclonal antibody, it was found that this antibody has an effect of improving the effect of high PTHrP-related hypercalcemia on central nerve system.

EP 1 197 225 A1

[CONSIDERATION]

1. Syndromes associated with malignancy

5 **[0119]** Examples of syndromes associated with malignancy include digestive system disorders (e.g. diarrhea, vomitition and nausea), proteometabolism abnormality (e.g. hypoalbuminemia), saccharometabolism abnormality (e.g. reduction of glucose tolerance and reduction of insulin secretion), lipid metabolism abnormality (e.g. hyperlipidemia and reduction of serum lipoprotein lipase ability), anorexia, hematological abnormality (e.g. anemia, thrombosis and DIC syndrome), electrolyte abnormality (e.g. hyponatremia, hypokalemia and hypercalcemia), immunodeficiency (e.g. infection disease), pain. The mechanism of onset of these syndromes associated with malignancy has not been clarified, but many of the symptoms of humoral hypercalcemia of malignancy overlap. As shown in the above Examples, when neutralizing antibody of PTHrP, a causal substance of hypercalcemia, is administered to model animals developing humoral hypercalcemia of malignancy, there can be observed improvements of symptoms such as not only normalization of blood calcium level but also increase in amount of movement, increase of food and water consumption, improvement of polyuria and normalization of vasopressin. As shown in Figure 4, it can hardly be explained that these improving effects are only the secondary effects by reduction (normalization) of blood calcium level, but it can be said that these effects are characteristic and specific functions attributable to PTHrP antagonists such as an anti-PTHrP antibody, irrespective of the effect of normalization of blood calcium level. From the above results, PTHrP is considered to be a causal substance of syndromes associated with malignancy, and various symptoms of the syndromes are considered to be provoked by means of a PTH/PTHrP receptor expressing in various organs. Therefore, it is considered that an active substance blocking a signal to a PTH/PTHrP receptor improves symptoms of syndromes associated with malignancy.

2. QOL improving agent

25 **[0120]** Various symptoms observed in syndromes associated with malignancy, humoral hypercalcemia of malignancy, primary hyperparathyroidism and secondary hyperparathyroidism, that is, symptoms such as digestive system disorders (e.g. diarrhea, vomitition and nausea), proteometabolism abnormality (e.g. hypoalbuminemia), saccharometabolism abnormality (e.g. reduction of glucose tolerance and reduction of insulin secretion), lipid metabolism abnormality (e.g. hyperlipidemia and reduction of serum lipoprotein lipase ability), anorexia, hematological abnormality (e.g. anemia, thrombosis and DIC syndrome), electrolyte abnormality (e.g. hyponatremia, hypokalemia and hypercalcemia), immunodeficiency (e.g. infection disease), and pain, significantly reduce QOL of a patient. It has been clarified that, among the above diseases, PTH or PTHrP acts as a causal substance in humoral hypercalcemia of malignancy, primary hyperparathyroidism and secondary hyperparathyroidism.

35 **[0121]** As described in "1. Syndromes associated with malignancy", when neutralizing antibody of PTHrP is administered to model animals developing humoral hypercalcemia of malignancy, there can be observed improvements of symptoms such as not only normalization of blood calcium level but also increase of amount of movement, increase of food and water consumption, improvement of polyuria and normalization of vasopressin. From the above results, PTHrP is considered to be a causal substance of syndromes associated with malignancy, and various symptoms of the syndromes are considered to be provoked by means of a PTH/PTHrP receptor expressing in various organs. Therefore, it is considered that an active substance blocking a signal to a PTH/PTHrP receptor improves the reduction of QOL in diseases such as syndromes associated with malignancy, humoral hypercalcemia of malignancy, primary hyperparathyroidism and secondary hyperparathyroidism.

3. Central nerve system

45 **[0122]** In syndromes associated with malignancy, humoral hypercalcemia of malignancy, primary hyperparathyroidism and secondary hyperparathyroidism, actions towards central nerve such as anorexia, mouth dryness and reduction of amount of movement can be observed. As stated above, improvements of symptoms such as increase of amount of movement, increase of food and water consumption, improvement of polyuria, normalization of vasopressin can be observed by administration of neutralizing antibody of PTHrP. Accordingly, PTHrP is considered as a causal substance of symptoms caused by central nerve, and these symptoms are considered to be provoked by means of a PTH/PTHrP receptor expressing in central nerve system. Therefore, an active substance blocking a signal to a PTH/PTHrP receptor is considered to improve symptoms caused by central nerve. Examples of central nerve system diseases include dysomnia, neuropathy (e.g. schizophrenia, manic-depressive psychosis, neurosis and psychophysiologic disorder), nervous symptom (e.g. vomiting, nausea, mouth dryness, anorexia and vertigo), brain metabolism abnormality, cerebral circulation abnormality, autonomic imbalance, and endocrine system abnormality with which central nervous system is associated, etc. PTHrP and a PTH/PTHrP receptor express in central nerve system (CNS), but

EP 1 197 225 A1

functions thereof have hardly been clarified yet. When localization of PTHrP mRNA in a rat brain was analyzed by in situ hybridization, it was found that the mRNA existed in the hippocampus, the granular cell layer of cerebellum, cerebral cortex and hypothalamus (Weaver et al., Mol Brain Res 28:296-301, 1995; Weir et al., Proc Natl Acad Sci USA 87: 108-112, 1990).

- 5 [0123] Moreover, the distribution of PTH/PTHrP receptor in rat brain matches with the distribution of PTHrP, and so it is assumed that PTHrP acts as a local autocrine/paracrine factor in central nervous system (CNS). An experiment regarding binding of PTH to a cell membrane fraction prepared from each site of rat brain teaches the order of binding strength as hypothalamus, cerebellum and cerebral cortex (Harvey et al., Peptides 14:1187-1191, 1993). Furthermore, it has been reported that arginine vasopressin (AVP) is released by PTHrP (1-34) stimulation of rat supraoptic nucleus (SON) slices, and that there is a possibility that PTHrP involves in homeostasis of in vivo water or electrolyte (Yamamoto et al., Endocrinology 139:383-388, 1998; Yamamoto et al., Endocrinology 138:2066-2072, 1997). Thus, the facts that PTHrP and a PTH/PTHrP receptor are widely distributed in brain and that there are common nervous symptoms and symptoms among the above diseases, suggest involvement of PTH or PTHrP as an onset cause of some of central nervous system diseases. Therefore, an active substance blocking a signal from PTH/PTHrP to the receptor is considered to improve central nervous system diseases.

4. Cytokine cascade

- 20 [0124] As stated above, as reports suggesting the possibility of crosstalk between PTH or PTHrP and cytokine, the following reports are known:

- 1) The values of IL-6 and TNF- α are high in a patient of primary hyperparathyroidism caused by high value of PTH (Grey A. et al., J Clin Endocrinol Metab 81:3450-5, 1996)
 2) When osteoblasts are stimulated by PTH or PTHrP in an in vitro system, expression of IL-6 and LfJ is promoted (Pollock JH. et al., J Bone Miner Res 11:754-9, 1996)
 3) A series of experiments with synovial cells showed that production of IL-6 is accentuated by stimulation with PTHrP, and that TNF- α and IL-1 β promote expression of PTHrP, and it was found that PTHrP is a member of pro-inflammatory cytokine cascade (Funk JL. et al., Endocrinology 138:2665-73, 1997; Funk JL. et al., J Clin Invest 101:1362-71, 1998)
 30 4) In cultured human vascular endothelial cells also, TNF- α and IL-1 β promote expression of PTHrP (Biochem Biophys Res Commun 249:339-343, 1998)

- 35 [0125] Moreover, it is known that not only generation of various cytokines but also that of PTHrP is induced in LPS-induced septicemia models. As shown in Examples, a neutralizing antibody of PTHrP has a life-prolonging effect in LPS-induced septicemia models.

- 40 [0126] Thus, PTH or PTHrP-cytokine cascade caused by induction of cytokine by PTHrP or induction of PTHrP by cytokine is considered to be involved in onset of pathology. Furthermore, since a life-prolonging effect was observed in LPS-induced septicemia models by blocking a cytokine-production inducing signal by PTHrP in various organs, an active substance blocking a signal from PTH/PTHrP to the receptor can be a therapeutic agent for septicemia, cachexia, inflammation, hemopathy such as hematopoietic system abnormality and leukaemia, calcium metabolism abnormality, and autoimmune disease such as rheumatism.

[REFERENCE EXAMPLE 1]

- 45 Preparation of hybridomas producing anti-PTHrP (1-34) mouse monoclonal antibody

- [0127] Hybridomas capable of producing a monoclonal antibody against human PTHrP (1-34) (SEQ ID NO: 75), #23-57-154 and #23-57-137-1, were prepared as follows (see Sato, K. et al., J. Bone Miner. Res. 8, 849-860, 1993). The amino acid sequence of the human PTHrP (1-34) is shown in SEQ ID NO: 75.

- 50 [0128] For use as an immunogen, PTHrP (1-34) (Peninsula) was conjugated with a carrier protein thyroglobulin using carbodiimide (Dojinn). The thyroglobulin-conjugated PTHrP (1-34) was dialyzed to obtain a solution having a protein concentration of 2 μ g/ml. The resulting solution was mixed with Freund's adjuvant (Difco) at a mixing ratio of 1:1 to give an emulsion. This emulsion was injected to 16 female BALB/C mice 11 times subcutaneously at the back or intraperitoneally at a dose level of 100 μ g/mouse for each injection, thereby immunizing the mice. For the priming immunization, Freund's complete adjuvant was used; while for the boosting immunization, Freund's incomplete adjuvant was used.

- 55 [0129] Each of the immunized mice was determined for its antibody titer in the serum in the following manner. That is, each of the mice was blood drawn via its tail vein, and the anti-serum is separated from the blood. The anti-serum

EP 1 197 225 A1

was diluted with a RIA buffer and mixed with ¹²⁵I-labeled PTHrP (1-34) to determine binding activity. The mice that were confirmed to have a sufficiently increased titer were injected with PTHrP (1-34) without a carrier protein intraperitoneally at a dose level of 50 µg/mouse for the final immunization.

[0130] Three days after the final immunization, the mouse is sacrificed and the spleen was removed therefrom. The spleen cells were subjected to cell fusion with mouse myeloma cell line P3x63Ag8U.1 in accordance with a conventional known method using 50% polyethylene glycol 4000. The fused cells thus prepared were seeded to each well of eighty-five 96-well plates at a density of 2 x 10⁴/well. Hybridomas were screened in HAT medium as follows.

[0131] The screening of hybridomas was performed by determining the presence of PTHrP-recognition antibodies in the culture supernatant of the wells in which cell growth had been observed in HAT medium, by solid phase RIA method. The hybridomas were collected from the wells in which binding ability to the PTHrP-recognition antibodies had been confirmed. The hybridomas thus obtained was suspended into RPMI-1640 medium containing 15% FCS supplemented with OPI-supplement (Sigma), followed by unification of the hybridomas by limiting dilution method. Thus, two types of hybridoma clones, #23-57-154 and #23-57-137-1, could be obtained, both which had a high binding ability to PTHrP (1-34).

[0132] Hybridoma clone #23-57-137-1 was designated as "mouse-mouse hybridoma #23-57-137-1", and has been deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) under the accession No. FERM BP-5631.

[REFERENCE EXAMPLE 2]

Cloning of DNAs encoding V-regions of mouse monoclonal antibody against human PTHrP (1-34)

[0133] Cloning of DNAs encoding the V-regions of a mouse monoclonal antibody against human PTHrP (1-34), #23-57-137-1, was performed in the following manner.

(1) Preparation of mRNA

[0134] mRNA from hybridoma #23-57-137-1 was prepared using Quick Prep mRNA Purification Kit (Pharmacia Biotech). That is, cells of hybridoma #23-57-137-1 were fully homogenized with an extraction buffer, and mRNA was isolated and purified therefrom on an oligo(dT)-Cellulose Spun Column in accordance with the instructions included in the kit. The resulting solution was subjected to ethanol precipitation to obtain the mRNA as a precipitate. The mRNA precipitate was dissolved in an elution buffer.

(2) Production and amplification of cDNA for gene encoding mouse H-chain V-region

(i) Cloning of cDNA for #23-57-137-1 antibody H-chain V-region

[0135] A gene encoding H-chain V-region of the mouse monoclonal antibody against human PTHrP was cloned by 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). The 5'-RACE method was performed using 5'-Ampli FINDER RACE Kit (CLONETECH) in accordance with the instructions included in the kit. In this method, the primer used for synthesis of cDNA was MHC2 primer (SEQ ID NO: 1) which is capable of hybridizing to mouse H-chain C-region. The above-prepared mRNA (about 2 µg), which was a template for the cDNA synthesis, was mixed with MHC2 primer (10 pmoles). The resulting mixture was reacted with a reverse transcriptase at 52° C for 30 minutes to effect the reverse transcription of the mRNA into cDNA.

[0136] The resulting reaction solution was added with 6N NaOH to hydrolyze any RNA remaining therein (at 85° C for 30 min.) and then subjected to ethanol precipitation to isolate and purify the cDNA as a precipitate. The purified cDNA was ligated to Ampli FINDER Anchor (SEQ ID NO: 42) at the 5' end by reacting with T4 RNA ligase at 37° C for 6 hours and additionally at room temperature for 16 hours. As the primers for amplification of the cDNA by PCR method, Anchor primer (SEQ ID NO: 2) and MHC-G1 primer (SEQ ID NO: 3) (S.T. Jones, et al., Biotechnology, 9, 88, 1991) were used.

[0137] The PCR solution comprised (per 50 µl) 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM MgCl₂, 2.5 units of TaKaRa Taq (Takara Shuzo Co., Ltd.), 10 pmoles Anchor primer, and 1 µl of the reaction mixture of the cDNA to which MHC-G1 primer and Ampli FINDER Anchor primer had been ligated, over which mineral oil (50 µl) was layered. The PCR was performed on Thermal Cycler Model 480J (Perkin Elmer) for 30 cycles under the conditions: 94° C for 45 sec.; 60° C for 45 sec.; and 72° C for 2 min.

EP 1 197 225 A1

(ii) Cloning of cDNA for #23-57-137-1 antibody L-chain V-region

[0138] A gene encoding L-chain V-region of the mouse monoclonal antibody against human PTHrP was cloned by 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). The 5'-RACE method was performed using 5'-Ampli Finder RACE Kit (CLONETECH) in accordance with the instructions included in the kit. In this method, oligo-dT primer was used as the primer for synthesizing cDNA. The above-prepared mRNA (about 2 µg), which was a template for the cDNA synthesis, was mixed with oligo-dT primer. The resulting mixture was reacted with a reverse transcriptase at 52° C for 30 min, to effect the reverse transcription of the mRNA into cDNA. The resulting reaction solution was added with 6N NaOH to hydrolyze any RNA remaining therein (at 65° C for 30 min.). The resulting solution was subjected to ethanol precipitation to isolate and purified the cDNA as a precipitate. The cDNA thus synthesized was ligated to Ampli FINDER Anchor at the 5' end by reacting with T4 RNA ligase at 37° C for 6 hours and additionally at room temperature for 16 hours.

[0139] A PCR primer MLC (SEQ ID NO: 4) was designed based on the conserved sequence of mouse L-chain λ chain C-region and then synthesized using 394 DNA/RNA Synthesizer (ABI). The PCR solution comprised (per 100 µl) 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM MgCl₂, 2.5 units of AmpliTaq (PERKIN ELMER), 50 pmoles of Anchor primer (SEQ ID NO: 2), and 1 µl of the reaction mixture of the cDNA to which MLC (SEQ ID NO: 4) and Ampli FINDER Anchor were ligated, over which mineral oil (50 µl) was layered. The PCR reaction was performed on Thermal Cycler Model 480J (Perkin Elmer) for 35 cycles under the conditions: 94° C for 45 sec.; 60° C for 45 sec.; and 72° C for 2 min.

(3) Purification and fragmentation of PCR products

[0140] Each of the DNA fragments amplified by PCR method described above was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products). For each of the H-chain V-region and the L-chain V-region, an agarose gel segment containing a DNA fragment of about 550 bp was excised from the gel. Each of the gel segments was subjected to purification of the DNA fragment of interest using GENECLONE II Kit (Bio101) in accordance with the instructions included in the kit. The purified DNA was precipitated with ethanol, and the DNA precipitate was dissolved in 20 µl of a solution containing 10 mM Tris-HCl(pH 7.4) and 1 mM EDTA. An aliquot (1 µl) of the DNA solution was digested with a restriction enzyme XmaI (New England Biolabs) at 37° C for 1 hour and further digested with a restriction enzyme EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA.

[0141] In this manner, two DNA fragments containing a gene encoding mouse H-chain V-region and a gene encoding mouse L-chain V-region, respectively, were obtained, both which had an EcoRI recognition sequence on the 5' end and an XmaI recognition sequence on the 3' end.

[0142] The EcoRI-XmaI DNA fragments containing a gene encoding mouse H-chain V-region and a gene encoding mouse L-chain V-region, respectively, were separately ligated to pUC19 vector that had been digested with EcoRI and XmaI at 16° C for 1 hour using DNA Ligation Kit ver.2 (Takara Shuzo Co., Ltd.) in accordance with the instructions included in the kit. An aliquot (10 µl) of the ligation mixture was added to 100 µl of a solution containing competent cells of E. coli, JM 109 (Nippon Gene Co., Ltd.). The cell mixture was allowed to stand on ice for 15 min., at 42° C for 1 min. and additionally for 1 min. on ice. The resulting cell mixture was added with 300 µl of SOC medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) and then incubated at 37° C for 30 min. The resulting cell solution was plated on LB agar medium or 2xYT agar medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) containing either 100 or 50 µg/ml of ampicillin, 0.1 mM of IPTG and 20 µg/ml of X-gal, and then incubated at 37° C overnight. In this manner, E. coli transformants were prepared.

[0143] The transformants were cultured at 37° C overnight in 2 ml of LB or 2xYT medium containing either 100 or 50 µg/ml of ampicillin. The cell fraction was applied to Plasmid Extractor PI-100 (Kurabo Industries, Ltd.) or QIAprep Spin Plasmid Kit (QIAGEN) to give a plasmid DNA. The plasmid DNA was sequenced as follows.

(4) Sequencing of genes encoding mouse antibody V-regions

[0144] The nucleotide sequence of the cDNA coding region carried on the plasmid was determined in DNA Sequencer 373A (ABI; Perkin-Elmer) using Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). M13 Primer M4 (Takara Shuzo Co., Ltd.) (SEQ ID NO: 5) and M13 Primer RV (Takara Shuzo Co., Ltd.) (SEQ ID NO: 6) were used as the primers for sequencing, and the nucleotide sequence was confirmed in the both directions.

[0145] The plasmid containing a gene encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 was designated as "MBC1H04", and the plasmid containing a gene encoding mouse L-chain V-region derived from hybridoma #23-57-137-1 was designated as "MBC1L24". The nucleotide sequences (including the corresponding amino

EP 1 197 225 A1

acids sequences) of the gene encoding the mouse #23-57-137-1 antibody-derived H-chain V-region in plasmid MBC1H04 and the gene encoding the mouse #23-57-137-1 antibody-derived L-chain V-region in plasmid MBC1H24 were shown in SEQ. ID Nos: 57 and 65, respectively. The amino acid sequences of the polypeptides for the H-chain V-region and the L-chain V-region were shown in SEQ. ID Nos: 46 and 45, respectively.

[0146] The E. coli strain containing the above plasmid MBC1H04 and the E. coli strain containing the above plasmid MBC1L24 were designated as "Escherichia coli JM109 (MBC1H04)" and "Escherichia coli JM109 (MBC1L24)", respectively. These E. coli strains have been deposited under the terms of the Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on August 15, 1996, under the Accession No. FERM BP-5628 for Escherichia coli JM109 (MBC1H04) and FERM BP-5627 for Escherichia coli JM109 (MBC1L24), respectively.

(5) Determination of CDRs of mouse monoclonal antibody #23-57-137-1 against human PTHrP

[0147] The H-chain V-region and the L-chain V-region have general structures similar to each other, each of which has four framework regions (FRs) linked through three hypervariable regions (i.e., complementarity determining regions; CDRs). The amino acid sequences of the FRs are relatively well conserved, while the amino acid sequence of the CDRs have an extremely high variability (Kabat, E.A. et al., "Sequence of Proteins of Immunological Interest", US Dept. Health and Human Services, 1983).

[0148] In view of these facts, the homology in amino acid between the V-regions of the mouse monoclonal antibody against human PTHrP was determined with reference to the database of amino acid sequences of antibodies established by Kabat et al. Thus, the CDRs of the V-regions were determined as shown in Table 1.

[0149] The amino acid sequences of CDRs 1-3 in the L-chain V-region are shown in SEQ ID Nos: 59 to 61, respectively; and the amino acid sequences of CDRs 1-3 in the H-chain V-region are shown in SEQ ID Nos: 62 to 64, respectively.

Table 1

V-region	SEQ ID NO.	CDR1	CDR2	CDR3
H-chain V-region	57	31-35	50-66	99-107
L-chain V-region	65	23-34	50-60	93-105

[REFERENCE EXAMPLE 3] Construction of Chimeric Antibody

(1) Construction of chimeric antibody H-chain

(i) Construction of H-chain V-region

[0150] To ligate to an expression vector carrying a genomic DNA of human H-chain C-region C γ 1, the cloned DNA encoding mouse H-chain V-region was modified by PCR method. A backward primer MBC1-S1 (SEQ ID NO: 7) was designed to hybridize to a DNA sequence encoding the 5' region of the leader sequence of the V-region and to have both a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and a HindIII-recognition sequence. A forward primer MBC1-a (SEQ ID NO: 8) was designed to hybridize to a DNA sequence encoding the 3' region of the J region and to have both a donor splice sequence and a BamHI-recognition sequence. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR solution comprised (per 50 μ l) 0.07 μ g of plasmid MBC1H04 as a template DNA, 50 pmoles of MBC1-a and 50 pmoles of MBC1-S1 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTPs in the buffer, over which 50 μ l of mineral oil was layered. The PCR was run for 30 cycles under the conditions: 94 $^{\circ}$ C for 1 min.; 55 $^{\circ}$ C for 1 min.; 72 $^{\circ}$ C for 2 min. The DNA fragments thus amplified by the PCR method were separated by agarose gel electrophoresis on a 3% Nu Sieve GTG Agarose (FMC Bio. Products).

[0151] Then, an agarose gel segment containing a DNA fragment of 437 bp was excised, and the DNA fragment was purified therefrom using GENECLAN II Kit (BIO101) in accordance with the instructions included in the kit. The purified DNA was collected by ethanol precipitation, and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. An aliquot (1 μ l) of the resulting DNA solution was digested with restriction enzymes BamHI and HindIII (Takara Shuzo Co., Ltd.) at 37 $^{\circ}$ C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA of interest.

[0152] The obtained HindIII-BamHI DNA fragment, which containing a gene encoding the mouse H-chain V-region, was subcloned into pUC19 vector that had been digested with HindIII and BamHI. The resulting plasmid was sequenced on DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV as primers and Dye Terminator

EP 1 197 225 A1

Cycle Sequencing Kit (Perkin-Elmer). As a result, a plasmid which carried a gene of correct nucleotide sequence encoding the mouse H-chain V-region derived from hybridoma #23-57-137-1 and had a HindIII-recognition sequence and a Kozak sequence on its 5' region and a BamHI-recognition sequence on its 3' region was obtained, which was designated as "MBC1H/pUC19".

(ii) Construction of H-chain V-region for preparation of cDNA-type of mouse-human chimeric H-chain

[0153] To ligate to cDNA of the human H-chain C-region C γ 1, the DNA encoding the mouse H-chain V-region constructed as described above was modified by PCR method. A backward primer MBC1HVS2 (SEQ ID NO: 9) for the V-region was designed to cause the replacement of the second amino acid (asparagine) of the sequence encoding the front part of the leader sequence of the H-chain V-region by glycine and to have a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and HindIII- and EcoRI-recognition sequences. A forward primer MBC1HVR2 (SEQ ID NO: 10) for the H-chain V-region was designed to hybridize to a DNA sequence encoding the 3' region of the J region, to encoding the 5' region of the C-region and to have Apal- and SmaI-recognition sequences.

[0154] The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR solution comprised (per 50 μ l) 0.6 μ g of plasmid MBC1H/pUC19 as a template DNA, 50 pmoles of MBC1HVS2 and 50 pmoles of MBC1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM of dNTPs in the buffer, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min.; 55° C for 1 min.; 72° C for 1 min. The DNA fragments amplified by the PCR reaction were separated by agarose gel electrophoresis on a 1% Sea Kern GTG Agarose (FMC Bio. Products). Then, an agarose gel segment containing a DNA fragment of 456 bp was excised and the DNA fragment was purified therefrom using GENECLEAN II Kit (Bio101) in accordance with the instructions included in the kit. The purified DNA was precipitated with ethanol and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0155] The resulting DNA solution (1 μ g) was digested with restriction enzymes EcoRI and SmaI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA. The obtained EcoRI-SmaI DNA fragment, which containing a gene encoding the mouse H-chain V-region, was subcloned into pUC19 vector that had been digested with EcoRI and SmaI. The resulting plasmid was sequenced on DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV, and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). As a result, a plasmid which contained a gene of correct nucleotide sequence encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 and had EcoRI- and HindIII-recognition sequences and a Kozak sequence on its 5' region and Apal- and SmaI-recognition sequences on its 3' region was obtained, which was designated as "MBC1Hv/pUC19".

(iii) Construction of expression vector for chimeric antibody H-chain

[0156] cDNA containing the DNA for human antibody H-chain C-region C γ 1 was prepared as follows. mRNA was prepared from a CHO cell into which both an expression vector DHFR- Δ E-RVh-PM-1-f (see WO 92/19759) encoding the genomic DNAs of humanized PM1 antibody H-chain V-region and human antibody H-chain C-region IgG1 (N. Takahashi et al., Cell 29, 671-679, 1982) and an expression vector RV1-PM1a (see WO 92/19759) encoding the genomic DNAs of humanized PM1 antibody L-chain V-region and human antibody L-chain κ chain C-region had been introduced. Using the mRNA, cDNA containing the humanized PM antibody H-chain V-region and the human antibody C-region C γ 1 was cloned by RT-PCR method, and then subcloned into plasmid pUC19 at the HindIII-BamHI site. After sequencing, a plasmid which had the correct nucleotide sequence was obtained, which was designated as "pRVh-PM1f-cDNA".

[0157] An expression vector DHFR- Δ E-RVh-PM-1-f in which both a HindIII site located between SV40 promoter and a DHFR gene and an EcoRI site located between EF-1 α promoter and a humanized PM1 antibody H-chain V-region gene had been deleted, was prepared for the construction of an expression vector for cDNA containing the humanized PM1 antibody H-chain V-region gene and the human antibody C-region C γ 1 gene.

[0158] The plasmid obtained (pRVh-PM1f-cDNA) was digested with BamHI, blunt-ended with Klenow fragment, and further digested with HindIII, thereby obtaining a blunt-ended HindIII-BamHI fragment. The blunt-ended HindIII-BamHI fragment was ligated to the above-mentioned HindIII site- and EcoRI site-deleted expression vector DHFR- Δ E-RVh-PM1-f that had been digested with HindIII and BamHI. Thus, an expression vector RVh-PM1f-cDNA was constructed which contained cDNA encoding the humanized PM1 antibody H-chain V-region and the human antibody C-region C γ 1.

[0159] The expression vector RVh-PM1f-cDNA containing the cDNA encoding the humanized PM1 antibody H-chain V-region and the human antibody C-region C γ 1 was digested with Apal and BamHI, and a DNA fragment containing the H-chain C-region was collected therefrom. The resulting DNA fragment was introduced into the plasmid MBC1Hv/pUC19 that had been digested with Apal and BamHI. The plasmid thus prepared was designated as "MBC1HcDNA".

EP 1 197 225 A1

pUC19". This plasmid contained cDNA encoding the mouse antibody H-chain V-region and the human antibody C-region C γ 1, and had EcoRI- and HindIII-recognition sequences on its 5' region and a BamHI-recognition sequence on its 3' region.

[0160] The plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment comprising a nucleotide sequence encoding the chimeric antibody H-chain. The resulting DNA fragment was introduced into an expression vector pCOS1 that had been digested with EcoRI and BamHI, thereby giving an expression vector for the chimeric antibody, which was designated as "MBC1HcDNA/pCOS1". Here, the expression vector pCOS1 was constructed using HEF-PMH-g γ1 (see WO 92/19759) by deleting therefrom an antibody genes by digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo Co., Ltd.)

[0161] For preparing a plasmid for the expression in a CHO cell, the plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to obtain a DNA fragment containing a gene for the chimeric antibody H-chain. The DNA fragment was then introduced into an expression plasmid pCHO1 that had been digested with EcoRI and BamHI to give an expression plasmid for the chimeric antibody, which was designated as "MBC1HcDNA/pCHO1". Here, the expression vector pCHO1 was constructed using DHFR-ΔE-rh-PM1-f (see WO 92/19759) by deleting therefrom an antibody gene by digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo Co., Ltd.)

(2) Construction of human L-chain C-region

(i) Preparation of cloning vector

[0162] To construct pUC19 vector containing a gene for human L-chain C-region, a HindIII site-deleted pUC 19 vector was prepared. pUC19 vector (2 μg) was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8 U of HindIII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The resulting digestion solution was extracted with phenol and chloroform, and then subjected to ethanol precipitation to collect the DNA of interest.

[0163] The DNA collected was reacted in 50 μl of a reaction solution containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 100 mM NaCl, 0.5 mM dNTPs and 6U of Klenow fragment (GIBCO BRL) at room temperature for 20 min., thereby rendering the terminal ends of the DNA blunt. This reaction mixture was extracted with phenol and chloroform and then subjected to ethanol precipitation to collect the vector DNA.

[0164] The vector DNA thus collected was reacted in 10 μl of a reaction solution containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (v/v) polyethylene glycol-8000 and 0.5 U of T4 DNA ligase (GIBCO BRL) at 16° C for 2 hours, to cause self-ligation of the vector DNA. The reaction solution (5 μl) was added to 100 μl of a solution containing competent cells of *E. coli*, JM109 (Nippon Gene Co., Ltd.), and the resulting solution was allowed to stand on ice for 30 min., at 42° C for 1 min., and additionally on ice for 1 min. SOC culture medium (500 μl) was added to the reaction solution and then incubated at 37° C for 1 hour. The resulting solution was plated on 2xYT agar medium (containing 50 μg/ml of ampicillin) on which X-gal and IPTG had been applied (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989), and then cultured at 37° C overnight, thereby obtaining a transformant.

[0165] The transformant was cultured in 2xYT medium (20 ml) containing ampicillin (50 μg/ml) at 37° C overnight. From the cell fraction of the culture medium, a plasmid DNA was isolated and purified using Plasmid Mini Kit (QIAGEN) in accordance with the instructions included in the kit. The purified plasmid was digested with HindIII. The plasmid that was confirmed to have a HindIII site-deletion was designated as "pUC19 Δ HindIII".

(ii) Construction of DNA encoding human L-chain λ chain C-region

[0166] Human antibody L-chain λ chain C-region is known to have at least four isotypes including Mcg⁺Ke⁺Oz⁻, Mcg⁺Ke⁺Oz⁻, Mcg⁺Ke⁺Oz⁺ and Mcg⁺Ke⁺Oz⁺ (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987). A search was made for a human antibody L-chain λ chain C-region homologous to the #23-57-137-1 mouse L-chain λ chain C-region from the EMBL database. As a result, it was found that the isotype Mcg⁺Ke⁺Oz⁻ of the human antibody L-chain λ chain (Accession No. X57819) (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987) showed the highest degree of homology to the #23-57-137-1 mouse L-chain λ chain C-region, with a 64.4% homology in terms of amino acid sequence and a 73.4% homology in terms of nucleotide sequence.

[0167] Then, a gene encoding the human antibody L-chain λ chain C-region was constructed by PCR method. The primers for the PCR were synthesized using 394 DNA/RNA Synthesizer (ABI). The synthesized primers were as follows: HLAMB1 (SEQ ID NO: 11) and HLAMB3 (SEQ ID NO: 13), both having a sense DNA sequence; and HLAMB2 (SEQ ID NO: 12) and HLAMB4 (SEQ ID NO: 14), both having an antisense DNA sequence; each primer containing a complementary sequence of 20-23 bp on the both terminal ends.

[0168] External primers HLAMB5 (SEQ ID NO: 15) and HLAMB6 (SEQ ID NO: 16) had sequences homologous to

EP 1 197 225 A1

the primers HLAMB1 and HLAMB4, respectively. HLAMBS contained EcoRI-, HindIII- and BlnI-recognition sequences, and HLAMBR contained an EcoRI-recognition sequence. In the first-round PCR reaction, the reactions between HLAMB1 and HLAMB2 and between HLAMB3 and HLAMB4 were performed. After the reactions were completed, both of the resulting PCR products were mixed in equivalent quantities, and then assembled in the second-round PCR reaction. The reaction solution was added with the external primers HLAMBS and HLAMBR. This reaction mixture was subjected to the third-round PCR reaction to amplify the full length DNA.

[0169] Each PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) in accordance with the instructions included in the kit. In the first-round PCR reaction, 100 μ l of either a reaction solution containing 5 pmoles of HLAMB1, 0.5 pmole of HLAMB2 and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) or a reaction solution containing 0.5 pmole of HLAMB3, 5 pmoles of HLAMB4 and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

[0170] In the second-round PCR reaction, a mixture of both the reaction solutions (50 μ l each) was used, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 3 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

[0171] In the third-round PCR reaction, the reaction solution to which the external primers HLAMBS and HLAMBR (50 pmoles each) were added was used. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

[0172] The DNA fragment obtained by the third-round PCR reaction was subjected to electrophoresis on a 3% low-melting agarose gel (NuSieve GTG Agarose, FMC), and separated and purified from the gel using GENECLAN II Kit (BIO101) in accordance with the instructions included in the kit.

[0173] The obtained DNA fragment was digested in a reaction solution (20 μ l) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 100 mM NaCl and 8U of EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform, and the DNA was collected therefrom by the ethanol precipitation.

The DNA was dissolved in a solution (8 μ l) containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0174] The above-prepared plasmid pUC19 Δ HindIII (0.8 μ g) was digested with EcoRI in the same manner as set forth above. The digestion solution was subjected to phenol/chloroform extraction and then ethanol precipitation, thereby giving a digested plasmid pUC19 Δ HindIII. The digested plasmid was reacted in a reaction solution (50 μ l) containing 50 mM Tris-HCl (pH 9.0), 1 mM MgCl₂ and alkaline phosphatase (E. coli C75; Takara Shuzo Co., Ltd.) at 37° C for 30 min. to dephosphorylate (i.e., BAP-treat) the plasmid. The reaction solution was subjected to phenol/chloroform extraction, and the DNA was collected therefrom by ethanol precipitation. The DNA thus obtained was dissolved in a solution (10 μ l) containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0175] The BAP-treated plasmid pUC19 Δ HindIII (1 μ l) was ligated to the above-obtained PCR product (4 μ l) using DNA Ligation Kit Ver.2 (Takara Shuzo Co., Ltd.). The resulting plasmid was introduced into a competent cell of E. coli, JM109, to give a transformant. The transformant was cultured overnight in 2xYT medium (2 ml) containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was isolated using QIAprep Spin Plasmid Kit (QIAGEN).

[0176] The obtained plasmid was sequenced for the cloned DNA part. The sequencing was performed on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). As a result, it was found that the cloned DNA had a 12-bp deletion therein. The plasmid was designated as "C Δ . Δ pUC19". Then, for making up for the deleted part, primers HCLMS (SEQ ID NO: 17) and HCLMR (SEQ ID NO: 18) were newly synthesized, and a DNA of correct sequence was reconstructed using these primers by PCR method.

[0177] In the first-round PCR reaction, the plasmid C Δ . Δ pUC19 having the DNA deletion therein was used as a template, and the reaction was performed with each of the primer sets of HLAMBS and HCLMS and HCLMS and HLAMB4. The PCR products were purified separately. In the second-round PCR reaction, the PCR products were assembled together. In the third-round PCR reaction, the reaction product of the second-round PCR reaction was added with external primers HLAMBS and HLAMB4 and amplified to give the full length DNA.

[0178] In the first-round PCR reaction, a reaction solution (100 μ l) containing 0.1 μ g of C Δ . Δ pUC19 as a template, either 50 pmoles of each of the primers HLAMBS and HCLMR or 50 pmoles of each of the primers HCLMS and HLAMB4, and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

[0179] The PCR products of the first-round PCR reaction, HLAMBS-HCLMR (236 bp) and HCLMS-HLAMB4 (147 bp), were subjected to electrophoresis separately on a 3% low-melting agarose gel to isolate the DNA fragments. The DNA fragments were collected and purified from the gels using GENECLAN II Kit (BIO101). In the second-round PCR reaction, 20 μ l of a reaction solution containing 40 ng of each of the purified DNA fragments and 1U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 25 μ l of mineral oil was layered. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

[0180] In the third-round PCR reaction, 100 μ l of a reaction solution containing 2 μ l of the reaction solution obtained by the second-round PCR reaction, 50 pmoles of each of external primers HLAMBS and HLAMB4 and 5U of TaKaRa

EP 1 197 225 A1

Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min., thereby obtaining a DNA fragment of 357 bp (the third PCR product). The DNA fragment was subjected to electrophoresis on a 3% low-melting agarose gel to isolate the DNA fragment. The resulting DNA fragment was collected and purified using GENECLEAN Kit (BIO101).

[0181] An aliquot (0.1 μ g) of the DNA fragment thus obtained was digested with EcoRI, and then subcloned into plasmid pUC19 Δ HindIII that had been BAP-treated. The resulting plasmid was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured overnight in 2 ml of 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was isolated and purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0182] The purified plasmid was sequenced on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). The plasmid that was confirmed to have the correct nucleotide sequence without any deletion was designated as "C ν pUC19".

(iii) Construction of gene encoding human L-chain κ chain C-region

[0183] A DNA fragment encoding the L-chain κ chain C-region was cloned from plasmid HEF-PM1k-gk (WO 92/19759) by PCR method. A forward primer HKAPS (SEQ ID NO: 19) was designed to contain EcoRI-, HindIII and BlnI-recognition sequences, and a backward primer HKAPA (SEQ ID NO: 20) was designed to contain an EcoRI-recognition sequence. These primers were synthesized on 394 DNA/RNA Synthesizer (ABI).

[0184] A PCR reaction was performed using 100 μ l of a reaction solution containing 0.1 μ g of plasmid HEF-PM1k-gk as a template, 50 pmoles of each of primers HKAPS and HKAPA and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.), over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min., thereby giving a PCR product of 360 bp. The DNA fragment was isolated and purified by electrophoresis on a 3% low-melting agarose, and then collected and purified using GENECLEAN II Kit (BIO101).

[0185] The thus obtained DNA fragment was digested with EcoRI, and then cloned into plasmid pUC19 (HindIII that had been BAP-treated). The resulting plasmid was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured overnight in 2 ml of 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0186] The purified plasmid was sequenced on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). The plasmid that was confirmed to have the correct nucleotide sequence was designated as "C ν pUC19".

(3) Construction of chimeric antibody L-chain expression vector

[0187] An expression vector for the chimeric #23-57-137-1 antibody L-chain was constructed. A gene encoding #23-57-137-1 L-chain V-region was ligated to the HindIII-BlnI site (located just in front of the human antibody C-region) of each of the plasmids C ν pUC19 and C ν pUC19, thereby obtaining pUC19 vectors that contained the DNAs encoding the chimeric #23-57-137-1 antibody L-chain V-region and either of the L-chain κ chain C-region or the L-chain κ region C-region, respectively. Each of the resulting vectors was then digested with EcoRI to separate the gene for the chimeric antibody L-chain. The gene was subcloned into HEF expression vector.

[0188] That is, a DNA fragment encoding #23-57-137-1 antibody L-chain V-region was cloned from plasmid MBC1L24 by PCR method. Primers used in the PCR method were separately synthesized using 394 DNA/RNA Synthesizer (ABI). A backward primer MBCCHL1 (SEQ ID NO: 21) was designed to contain a HindIII-recognition sequence and a Kozak sequence (Kozak, M. et al., J. Mol. Biol. 196, 947-950, 1987), and a forward primer MBCCHL3 (SEQ ID NO: 22) was designed to contain BglII- and RcoRI-recognition sequences.

[0189] The PCR reaction was performed using 100 μ l of a reaction solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.1 μ g MBC1L24, 50 pmoles of each of primers MBCCHL1 and MBCCHL3 and 1 μ l of AmpliTaq (PERKIN ELMER), over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 45 sec., 60° C for 45 sec. and 72° C for 2 min.

[0190] A PCR product of 444 bp was electrophoresed on a 3% low-melting agarose gel, and collected and purified using GENECLEAN II Kit (BIO101). The purified PCR product was dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR product (1 μ l) was digested in 20 μ l of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 8U of EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was subjected to phenol/chloroform extraction, and the DNA of interest was collected therefrom by ethanol precipitation. The DNA was dissolved in 8 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

EP 1 197 225 A1

[0191] In the same manner, plasmid pUC19 (1 µg) was digested with HindIII and EcoRI, and subjected to phenol/chloroform extraction and then ethanol precipitation. The obtained digested plasmid was BAP-treated with alkaline phosphatase (E. coli C75; Takara Shuzo Co., Ltd.). The resulting reaction solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0192] The BAP-treated plasmid pUC19 (1 µl) was ligated to the above-obtained PCR product (4 µl) using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.). The resulting plasmid was introduced into a competent cell of E. coli, JM109 (Nippon Gene Co., Ltd.), in the same manner as set forth above, to form a transformant. The transformant was plated on 2xYT agar medium containing 50 µg/ml of ampicillin and cultured at 37°C overnight. The resulting transformant was then cultured at 37°C overnight in 2 ml of 2xYT medium containing 50 µg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). After determining the nucleotide sequence, the plasmid that was confirmed to have the correct nucleotide sequence was designated as "CHL/pUC19".

[0193] Each of plasmids Cλ/pUC19 and Cκ/pUC19 (1 µg each) was digested in 20 µl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2U of BlnI (Takara Shuzo Co., Ltd.) at 37°C for 1 hour. The digestion solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was BAP-treated at 37°C for 30 min. The reaction solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0194] The plasmid CHL/pUC19 (8 µg) that contained DNA encoding #23-57-137-1 L-chain V-region was digested with HindIII and BlnI in the same manner as set forth above to give a DNA fragment of 409 bp. The DNA fragment was electrophoresed on a 3% low-melting agarose gel, and then collected and purified from the gel using GENECLON II Kit (BIO101). The DNA was dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0195] The DNA for L-chain V-region DNA (4 µl) was subcloned into 1 µl of each of the BAP-treated plasmids Cλ/pUC19 and Cκ/pUC19, and then introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured overnight in 3 ml of 2xYT medium containing 50 µg/ml of ampicillin. From the cell fraction, the plasmid was isolated and purified using QIAprep Spin Plasmid Kit (QIAGEN). The two plasmids thus prepared were designated as "MBC1L(λ)/pUC19" and "MBC1L(κ)/pUC19", respectively.

[0196] Each of plasmids MBC1L(λ)/pUC19 and MBC1L(κ)/pUC19 was digested with EcoRI and then subjected to electrophoresis on a 3% low-melting agarose gel. A DNA fragment of 743 bp was isolated and purified from the gel using GENECLON II Kit (BIO101), and then dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0197] An expression vector (plasmid HEF-PM1k-gk) (2.7 µg) was digested with EcoRI and then extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA fragment was BAP-treated, and then subjected to electrophoresis on a 1% low-melting agarose gel. From the gel, a DNA fragment of 6561 bp was isolated and purified therefrom using GENECLON II Kit (BIO101). The purified DNA fragment was dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0198] BAP-treated HEF vector (2 µl) was ligated to an EcoRI fragment (3 µl) of each of plasmid MBC1L(λ)/pUC19 and MBC1L(κ)/pUC19. The ligation product was introduced into a competent cell of E. coli, JM 109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 µg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0199] The purified plasmid was digested in 20 µl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2 U of PvuI (Takara Shuzo Co., Ltd.) at 37°C for 1 hour. This reaction gave digestion fragments of 5104/2195 bp if the fragment was inserted in the correct orientation, or gave digestion fragments of 4378/2926 bp if the fragment was inserted in the reverse orientation. The plasmid that was confirmed to have the fragment in the correct orientation was designated as "MBC1L(λ)/neo" for plasmid MBC1L(λ)/pUC19 or "MBC1L(κ)/neo" for plasmid MBC1L(κ)/pUC19.

(4) Transfection of COS-7 cell

[0200] To evaluate the antigen-binding activity and the neutralizing activity of the chimeric antibodies, the expression plasmids prepared above were separately expressed transiently in a COS-7 cell.

[0201] The transient expression of the chimeric antibodies was performed using each of the combinations of plasmids MBC1LhcDNA/pCOS1 and MBC1L(λ)/neo and plasmids MBC1LhcDNA/pCOS1 and MBC1L(κ)/neo, by co-transfecting a COS-7 cell with the plasmids by electroporation using Gene Pulser (Bio Rad). That is, the plasmids (10 µg each) were added to a COS-7 cell suspension (0.8 ml; 1 × 10⁷ cells/ml) in PBS(-). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 2 µF to cause electroporation. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in DMEM medium (GIBCO) containing 2% Ultra Low IgG fetal calf serum (GIBCO), and then cultured using a 10-cm culture dish in a CO₂ incubator. After culturing for 72 hours,

EP 1 197 225 A1

a culture supernatant was collected and centrifuged to remove cell debris, and was provided for use as a sample for the subsequent ELISA.

[0202] In this procedure, the purification of the chimeric antibody from the COS-7 cell culture supernatant was performed using AffiGel Protein A MAPSII Kit (Bio Rad) in accordance with the instructions included in the kit.

(5) ELISA

(i) Determination of antibody concentration

[0203] An ELISA plate for determining antibody concentration was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μ l of a coating buffer (0.1 M NaHCO₃, 0.02% NaN₃) supplemented with 1 μ g/ml of goat anti-human IgG antibody (TAGO), and then blocked with 200 μ l of a dilution buffer [50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, 0.05% Tween 20, 0.02% NaN₃, 1% bovine serum albumin (BSA); pH 7.2]. Each well of the plate was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the chimeric antibodies had been expressed, or added with each of the serial dilutions of each of the chimeric antibodies per se in a purified form. The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Each well of the plate was then added with 100 μ l of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After the plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20, each well was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad) to determine the antibody concentration. In this determination, Hu IgG1 λ Purified (The Binding Site) was used as the standard substance.

(ii) Determination of antigen-binding ability

[0204] An ELISA plate for the determination of antigen-binding ability was prepared as follows. Each well of a 96-well ELISA plate was coated with 100 μ l of a coating buffer supplemented with 1 μ g/ml of human PTHrP (1-34) (Peptide Research Institute), and then blocked with 200 μ l of a dilution buffer. Each well was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the chimeric antibodies had been expressed, or added with each of the serial dilutions of each of the chimeric antibodies per se in a purified form. After the plate was incubated at room temperature and washed with PBS-Tween 20, each well of the plate was added with 100 μ l of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After the plate was incubated at room temperature and washed with PBS-Tween 20, each well of the plate was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad).

[0205] As a result, it was found that the chimeric antibodies had an ability to bind to human PTHrP (1-34) and the cloned mouse antibody V-regions had the correct structures (FIG. 5). It was also found that there was no difference in the ability to bind to PTHrP (1-34) between the chimeric antibody with L-chain λ chain C-region and the chimeric antibody with L-chain κ chain C-region. Therefore, the humanized antibody L-chain λ chain was used for construction of the L-chain C-region of the humanized antibody.

(6) Establishment of CHO cell line capable of stable production of chimeric antibodies

[0206] To establish a cell line capable of producing the chimeric antibodies stably, the above-prepared expression plasmids were introduced into CHO cells (DXB11).

[0207] For the establishment of a cell line capable of producing the chimeric antibodies stably, either of the following combinations of the expression plasmids for CHO cell was used: MBC1HcDNA/pCHO1 and MBC1L(λ)/neo; and MBC1HcDNA/pCHO1 and MBC1L(κ)/neo. A CHO cell was co-transfected with the plasmids by electroporation using Gene Pulser (Bio Rad) as follows. The expression vectors were separately cleaved with a restriction enzyme PvuI to give linear DNAs. The resulting DNAs were extracted with phenol and chloroform and collected by precipitation with ethanol. The plasmid DNAs thus prepared were subjected to electroporation. That is, each of the plasmid DNAs (10 μ g each) was added to 0.8 ml of a cell suspension of CHO cells in PBS(-) (1×10^7 cells/ml). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μ F. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in MEM- α medium (GIBCO) containing 10% fetal calf serum (GIBCO). The resulting suspension was cultured using three 96-well plates (Falcon) in a CO₂ incubator. On the day following the culturing being started, the medium was replaced by a selective medium [ribonucleoside- or deoxyribonucleoside-free MEM- α medium (GIBCO) containing 10% fetal calf serum (GIBCO) and 500 mg/ml of GENETICIN (G418Sulfate; GIBCO)]. From the culture medium, cells into which the antibody gene was introduced were selected. The selective medium is replaced by a fresh one. About two weeks after the medium replacement, the cells were observed under a

EP 1 197 225 A1

microscope. When a satisfactory cell growth was observed, the amount of the antibodies produced was determined by ELISA as set forth above. Among the cells, those cells which produced a larger amount of antibodies were screened. [0208] Then, the culturing of the established cell line capable of stable production of the antibodies was scaled up in a roller bottle using ribonucleoside- or deoxyribonucleoside-free MEM medium containing 2% Ultra Low IgG fetal calf serum. On day 3 and day 4 of the culturing, the culture supernatant was collected and then filtered on a 0.2- μ m filter (Millipore) to remove cell debris therefrom.

[0209] Purification of the chimeric antibodies from the CHO cell culture supernatant was performed using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with the instructions included in the kit. The purified chimeric antibodies were provided for use as samples for the determination of neutralizing activity and for the examination of therapeutic efficacy in hypercalcemic model animals. The concentration and the antigen-binding activity of the purified chimeric antibodies were determined using the same ELISA system as set forth above.

[REFERENCE EXAMPLE 4] Construction of humanized antibody

(1) Construction of humanized antibody H-chain

(i) Construction of humanized H-chain V-region

[0210] A humanized #23-57-137-1 antibody H-chain was produced by CDR-grafting technique by means of PCR method. For the production of a humanized #23-57-137-1 antibody H-chain (version "a") having FRs derived from human antibody S31679 (NBRF-PDB; Cuisinier, A. M. et al., Eur. J. Immunol., 23, 110-118, 1993), the following six PCR primers were used: CDR-grafting primers: MBC1HGP1 (SEQ ID NO: 23) and MBC1HGP3 (SEQ ID NO: 24) (both containing a sense DNA sequence) and MBC1HGP2 (SEQ ID NO: 25) and MBC1HGP4 (SEQ ID NO: 26) (both containing an antisense DNA sequence), all of which containing a 15-21 bp complementary sequence on both terminal ends thereof; and external primers: MBC1HVS1 (SEQ ID NO: 27) and MBC1HVR1 (SEQ ID NO: 28) having a homology to the CDR-grafting primers MBC1HGP1 and MBC1HGP4, respectively.

[0211] The CDR-grafting primers MBC1HGP1, MBC1HGP2, MBC1HGP3 and MBC1HGP4 were separated on an urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989), and extracted therefrom by crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) in the following manner.

[0212] Each of the CDR-grafting primers (1 nmole) was separated on a 6% denatured polyacrylamide gel to give DNA fragments. From the resulting DNA fragments, a DNA fragment having a desired length was identified on a silica gel thin plate by irradiation of UV ray and then collected therefrom by crush-and-soak method. The resulting DNA was dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.). The PCR reaction solution (100 μ l) comprised 1 μ l of each of the above-mentioned CDR-grafting primers MBC1HGP1, MBC1HGP2, MBC1HGP3 and MBC1HGP4, 0.25 mM dNTPs and 2.5U of TaKaRa Ex Taq in the buffer. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The resulting reaction solution was added with the external primers MBC1HVS1 and MBC1HVR1 (50 pmole each). Using this reaction mixture, the PCR reaction was run for additional 30 cycles under the same conditions. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 4% Nu Sieve GTG agarose (FMC Bio. Products).

[0213] An agarose segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLONE Kit (BIO101) in accordance with the instructions included in the kit. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The resulting PCR reaction mixture was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII, and subsequently the nucleotide sequence of the resulting plasmid was determined. A plasmid having the correct nucleotide sequence was designated as "hMBCHv/pUC19".

(ii) Construction of H-chain V-region of Humanized H-chain cDNA

[0214] To ligate to cDNA for humanized H-chain C-region Cy1, the DNA for the humanized H-chain V-region constructed in the above step was modified by PCR method. For the PCR method, a backward primer MBC1HVS2 was designed to hybridize to the sequence encoding the 5' region of the leader sequence for the V-region and to have a Kozak consensus sequence (Kozak et al., J. Mol. Biol. 196, 947-950, 1987) and HindIII- and EcoRI-recognition sequences; and a forward primer MBC1HVR2 was designed to hybridize to both the DNA sequence encoding the 3' region of the J region and the DNA sequence encoding the 5' region of the C-region and to have ApaI- and SmaI-recognition sequences.

[0215] The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended

EP 1 197 225 A1

thereto. The PCR reaction solution comprised 0.4 µg of hMBCHv/pUC19 as a DNA template, 50 pmoles of each of MBC1HVS2 and MBC1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTPs in the buffer. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

[0216] A gel segment containing a DNA fragment of 456 bp was excised, and the DNA fragment was purified therefrom using GENECLONE II Kit (BIO101) in accordance with the instructions included in the kit. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction solution thus obtained was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with EcoRI and SmaI, and then the resulting plasmid was sequenced. As a result, a plasmid was obtained which contained a DNA encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 and also contained EcoRI- and HindIII-recognition sequences and a Kozak sequence on the 5' region and ApaI- and SmaI-recognition sequences on the 3' region, which was designated as "hMBC1Hv/pUC19".

(2) Construction of expression vector for humanized antibody H-chain

[0217] Plasmid RVh-PM1f-cDNA carrying a cDNA sequence for hPM1 antibody H-chain was digested with ApaI and BamHI to give a DNA fragment containing a DNA fragment containing a DNA encoding the H-chain C-region. The DNA fragment was introduced into plasmid hMBC1Hv/pUC19 that had been digested with ApaI and BamHI. The obtained plasmid was designated as "hMBC1HcDNA/pUC19". This plasmid contained both a DNA encoding the humanized #23-57-137-1 antibody H-chain V-region and a DNA encoding the human H-chain C-region C γ1 and had EcoRI- and HindIII-recognition sequences on the 5' region and a BamHI-recognition sequence on the 3' region. The nucleotide sequence and the corresponding amino acid sequence of the humanized H-chain version "a" carried on the plasmid hMBC1HcDNA/pUC19 are shown in SEQ ID NO: 58 and SEQ ID NO: 56, respectively.

[0218] The plasmid hMBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment containing a DNA encoding the H-chain. The DNA fragment was introduced into expression plasmid pCOS1 that had been digested with EcoRI and BamHI. As a result, an expression plasmid for a humanized antibody was obtained, which was designated as "hMBC1HcDNA/pCOS1".

[0219] To produce a plasmid used for expression in a CHO cell, plasmid hMBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment containing a DNA encoding the H-chain. The DNA fragment was introduced into expression vector pCHO1 that had been digested with EcoRI and BamHI. As a result, an expression plasmid for the humanized antibody was obtained, which was designated as "hMBC1HcDNA/pCHO1".

(3) Construction of L-chain hybrid V-region

(i) Preparation of FR1,2/FR3,4 hybrid antibody

[0220] A gene for the FR hybrid L-chain having both FRs from a humanized antibody and FRs from a mouse (chimeric) antibody was constructed, and evaluated each region for the humanization. In this step, a hybrid antibody having FR1 and FR2 both derived from a human antibody and FR3 and FR4 both derived from a mouse antibody was prepared by utilizing the AflII restriction site located on CDR2.

[0221] Plasmids MBC1L(λ)/neo and hMBC1L(λ)/neo (10 µg each) were separately digested in 100 µl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 10 U of AflII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The reaction solutions were subjected to electrophoresis on a 2% low-melting agarose gel, thereby giving DNA fragments of 6282 bp (referred to as "c1") and 1022 bp (referred to as "c2") from the plasmid MBC1L(λ)/neo or DNA fragments of 6282 bp (referred to as "h1") and 1022 bp (referred to as "h2") from the plasmid hMBC1L(λ)/neo. These DNA fragments were collected and purified from the gels using GENECLONE II Kit (BIO101).

[0222] Each of the c1 and h1 fragments (1 µg each) was BAP-treated. The DNA fragment was extracted with phenol and chloroform, collected by ethanol precipitation, and then dissolved in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0223] The BAP-treated c1 and h1 DNA fragments (1 µl each) were ligated to the h2 and c2 DNA fragments (4 µl each), respectively, (at 4° C overnight). Each of the ligation products was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 µg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0224] The purified plasmid was digested in 20 µl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, and either 2U of ApaI (Takara Shuzo Co., Ltd.) or 8U of BamHI (Takara Shuzo Co., Ltd.) and HindIII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. It was expected that if the c1-h2 was ligated correctly, this digestion reaction would give fragments of 5560/1246/498 bp (by the ApaI digestion) or fragments of 7134/269 bp (by the

EP 1 197 225 A1

BamHI/HindIII digestion). Based on this expectation, the desired plasmids were identified.

[0225] The expression vector encoding the human FR1.2/mouse FR3.4 hybrid antibody L-chain was designated as "h/mBC1L(λ)/neo". On the other hand, since a clone for the h1-c1 could not be obtained, recombination on a pUC vector was performed and then the resulting recombinant product was cloned into a HEF vector. In this procedure, plasmid hMBC1Laλ/pUC19, which contained DNA encoding a humanized antibody L-chain V-region without any amino acid replacements, and plasmid hMBC1Ldλ/pUC19, which contained a DNA encoding a humanized antibody L-chain V-region with an amino acid replacement at the 91-position amino acid tyrosine in FR3 (i.e., the 87th amino acid in accordance with The Kabat's prescription) by isoleucine, were used as templates.

[0226] Plasmids MBC1L(λ)/pUC19, hMBC1Laλ/pUC19 and hMBC1Ldλ/pUC19 (10 μl each) were separately digested in 30 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA, 16U of HindIII and 4U of AluI at 37° C for 1 hour. The reaction solutions were separately subjected to electrophoresis on a 2% low-melting agarose gel, thereby giving a DNA fragment of 215 bp from plasmid MBC1L(λ)/pUC19 (referred to as "c2") and a DNA fragment of 3218 bp from each of plasmids hMBC1Laλ/pUC19 and hMBC1Ldλ/pUC19 (referred to as "ha1" and "hd1", respectively). These DNA fragments were collected and purified using GENECLEANII Kit (BIO101).

[0227] Each of the ha1 and hd1 fragments was ligated to the c2' fragment and then introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). The plasmids thus prepared were designated as "m/hMBC1Laλ/pUC19" for the ha1 fragment-containing plasmid and "m/hMBC1Ldλ/pUC19" for the hd1 fragment-containing plasmid.

[0228] Each of the obtained plasmids m/hMBC1Laλ/pUC19 and m/hMBC1Ldλ/pUC19 was digested with EcoRI. The DNA fragment of 743 bp was electrophoresed on a 2% low-melting agarose gel, and then collected and purified therefrom using GENECLEANII Kit (BIO101). The resulting DNA fragment was dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0229] Each of the DNA fragments (4 μl each) was ligated to the above-obtained BAP-treated HEF vector (1 μl). The ligation product was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0230] Each of the purified plasmids was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2U of PvuI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. It was expected that if the DNA fragment was inserted in the plasmid in a correct orientation, this digestion would give digestion fragments of 5104/2195 bp, whereas if the DNA fragment is inserted in the plasmid in the reverse orientation, this digestion would give digestion fragments of 4378/2926 bp. The plasmid DNA was identified based on the expectation. The plasmids thus obtained were expression vectors encoding mouse FR1.2/human FR3.4 hybrid antibody L-chain, which were designated as expression vectors "m/hMBC1Laλ/neo" and "m/hMBC1Ldλ/neo", respectively.

(ii) Preparation of FR1/FR2 hybrid antibody

[0231] An FR1/FR2 hybrid antibody was prepared in the same manner as set forth above utilizing a SnaBI restriction site located on CDR1.

[0232] Plasmids MBC1L(λ)/neo and h/mMBC1L(λ)/neo (10 μg each) were separately digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 6U of SnaBI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The resulting reaction solutions were further digested in 50 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 0.01% (w/v) of BSA and 6U of PvuI at 37° C for 1 hour.

[0233] The resulting reaction solutions were separately subjected to electrophoresis on a 1.5% low-melting agarose gel, thereby giving DNA fragments of 4955 bp (m1) and 2349 bp (m2) from the plasmid MBC1L(λ)/neo and DNA fragments of 4955 bp (hm1) and 2349 bp (hm2) from the plasmid h/mMBC1L(λ)/neo. These DNA fragments were collected and purified from the gels using GENECLEANII Kit (BIO101). Each of the DNA fragments obtained was dissolved in 40 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0234] The m1 and hm1 fragments (1 μl each) were ligated to the hm2 and m2 fragments (4 μl each), respectively. Each of the resulting ligation products was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant obtained was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

[0235] Each of the purified plasmids was digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT and either 8U of Apal (Takara Shuzo Co., Ltd.) or 2U of ApaI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour.

EP 1 197 225 A1

[0236] It was expected that if the fragments were ligated correctly, the digestion reaction would give a fragment of 7304 bp (by the *Apal* digestion) or fragments of 5560/1246/498 bp (by the *Apal* digestion) for m1-hm2, and would give fragments of 6538/766 bp (by the *Apal* digestion) or fragments of 3535/2025/1246/498 bp (by the *Apal* digestion) for hm1-m2. Based on this expectation, the plasmids were identified. As a result, an expression vector encoding a human FR1/mouse FR2,3,4 hybrid antibody L-chain (designated as "hmmMBC1L(λ)/neo") and an expression vector encoding a mouse FR1/human FR2/mouse FR3,4 hybrid antibody L-chain (designated as "mhmMBC1L(λ)/neo") were obtained.

(4) Construction of humanized antibody L-chain

[0237] A humanized #23-57-137-1 antibody L-chain was prepared by CDR-grafting technique by means of PCR method. For the preparation of a humanized #23-57-137-1 antibody L-chain (version "a") that contained FR1, FR2 and FR3 derived from human antibody HSU03868 (GEN-BANK, Deltos M. et al., *Scand. J. Immunol.*, 39, 95-103, 1994) and FR4 derived from human antibody S26755 (NBRF-PDB), six PCR primers were used.

[0238] The six primers were as follows: CDR-grafting primers MBC1LGP1 (SEQ ID NO: 29) and MBC1LGP3 (SEQ ID NO: 30), both having a sense DNA sequence, CDR-grafting primers MBC1LGP2 (SEQ ID NO: 31) and MBC1LGP4 (SEQ ID NO: 32), both having an antisense DNA sequence, all of which had a 15-21 bp complementary sequence on the both terminal ends; and external primers MBC1LVS1 (SEQ ID NO: 33) and MBC1LVR1 (SEQ ID NO: 34) having a homology to the CDR-grafting primers MBC1LGP1 and MBC1LGP4, respectively.

[0239] The CDR-grafting primers MBC1LGP1, MBC1LGP2, MBC1LGP3 and MBC1LGP4 were separated on a urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) and extracted therefrom by crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989).

[0240] Each of the CDR-grafting primers (1 nmole each) was separated on a 6% denatured polyacrylamide gel. The identification of the DNA fragment of a desired length was performed on a silica gel thin plate by irradiation of UV ray. The desired DNA fragment was collected from the gel by crush-and-soak method. The collected DNA fragment was dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

[0241] The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR reaction solution comprised (per 100 μ l) 1 μ l of each of the CDR-grafting primers MBC1LGP1, MBC1LGP2, MBC1LGP3 and MBC1LGP4, 0.25 mM dNTPs, 2.5U of TaKaRa Ex Taq in the buffer. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 55° C for 1 min., and 72° C for 1 min. The resulting reaction mixture was added with 50 pmoles of each of the external primers MBC1LVS and MBC1LVR1. Using this reaction mixture, the PCR reaction was run for additional 30 cycles under the same conditions. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

[0242] An agarose segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLANII Kit (BIO101) in accordance with the instructions included in the kit. The PCR reaction mixture thus obtained was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII. The resulting plasmid was sequenced. The plasmid thus prepared was designated as "hMBCL/pUC19". In this plasmid, however, the 104-position amino acid (corresponding to the 96th amino acid in accordance with the Kabat's prescription) of CDR4 was replaced by arginine. For the correction of this amino acid to tyrosine, a correction primer MBC1LGP10R (SEQ ID NO: 35) was designed and synthesized. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR reaction solution comprised (per 100 μ l) 0.6 μ g of the plasmid hMBCL/pUC19 as a template DNA, 50 pmoles of each of the primers MBC1LVS1 and MBC1LGP10R, 2.5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and 0.25 mM dNTPs in the buffer, over which mineral oil (50 μ l) was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 55° C for 1 min., and 72° C for 1 min. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

[0243] A gel segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLANII Kit (BIO101) in accordance with the instructions included in the kit. The PCR reaction mixture thus prepared was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII.

[0244] The plasmid was sequenced using M13 Primer M4 and M13 Primer RV. As a result, it was confirmed that the plasmid had the correct sequence. The plasmid was then digested with HindIII and BlnI, and a DNA fragment of 416 bp was separated by electrophoresis on a 1% agarose gel. The DNA fragment was purified using GENECLANII Kit (BIO101) in accordance with the instructions included in the kit, and then introduced into plasmid C λ /pUC19 that had been digested with HindIII and BlnI. The resulting plasmid was designated as "hMBC1La λ /pUC19". This plasmid was digested with EcoRI to give a DNA fragment encoding humanized L-chain. The DNA fragment was introduced into plasmid pCOS 1 so that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter.

EP 1 197 225 A1

The plasmid thus obtained was designated as "hMBC1La λ pCOS1". The DNA sequence (including the corresponding amino acid sequence) of the humanized L-chain version "a" is shown in SEQ ID NO: 66. The amino acid sequence of the version "a" is also shown in SEQ ID NO: 47.

[0245] A humanized L-chain version "b" was prepared using mutagenesis by PCR method. The version "b" was designed such that the 43-position amino acid glycine (corresponding to the 43th amino acid in accordance with the Kabat's prescription) was replaced by proline and the 49-position amino acid lysine (corresponding to the 49th amino acid in accordance with the Kabat's prescription) by aspartic acid in the version "a". The PCR reaction was performed using plasmid hMBC1 La λ pUC19 as a template and a mutagenic primer MBC1LGP5R (SEQ ID NO: 36) and a primer MBC1LUS1. The DNA fragment obtained was digested with BamHI and HindIII, and the digestion fragment was subcloned into the BamHI-HindIII site of pUC19. After sequencing, the plasmid was digested with HindIII and AflII, and the resulting digestion fragment was ligated to plasmid hMBC1 La λ pUC19 that had been digested with HindIII and AflII.

[0246] The thus obtained plasmid was designated as "hMBC1Lb λ pUC19". This plasmid was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated as "hMBC1Lb λ pCOS1".

[0247] A humanized L-chain version "c" was prepared using mutagenesis by PCR method. The version "c" was designed such that the 84-position amino acid serine (corresponding to the 80th amino acid in accordance with the Kabat's prescription) was replaced by proline. The PCR reaction was performed using plasmid hMBC1La λ pUC19 as a template and a mutagenic primer MBC1LGP6S (SEQ ID NO: 37) and a primer M13 Primer RV. The DNA fragment obtained was digested with BamHI and HindIII and then subcloned into pUC19 that had been digested with BamHI and HindIII.

[0248] After sequencing, the plasmid was digested with BstPI and Aor51HI, and the resulting DNA fragment was ligated to plasmid hMBC1La λ pUC19 that had been digested with BstPI and Aor51HI. The plasmid thus obtained was designated as "hMBC1Lc λ pUC19". This plasmid was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated as "hMBC1Lc λ pCOS1".

[0249] Humanized L-chain versions "d", "e" and "f" were also prepared using mutagenesis by PCR method. The versions "d", "e" and "f" were designed such that the 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine in the versions "a", "b" and "c", respectively. For each of the versions "d", "e" and "f", a PCR reaction was performed using each of plasmid hMBC1La λ pCOS1 (for version "d"), hMBC1Lb λ pCOS1 (for version "e") and hMBC1Lc λ pCOS1 (for version "f"), respectively, as a template, a mutagenic primer MBC1LGP11R (SEQ ID NO: 38) and a primer M-S1 (SEQ ID NO: 44). The DNA fragment thus obtained was digested with BamHI and HindIII and then subcloned into pUC19 that had been digested with BamHI and HindIII. After sequencing, the plasmid was digested with HindIII and BlnI, and the resulting digestion fragment was ligated to plasmid C λ pUC19 that had been digested with HindIII and BlnI.

[0250] The thus obtained plasmids were respectively designated as "hMBC1Ld λ pUC19" (for version "d"), "hMBC1Le λ pUC19" (for version "e") and "hMBC1Lf λ pUC19" (for version "f"). Each of these plasmids was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter of the plasmid. The plasmids thus obtained were respectively designated as "hMBC1Ld λ pCOS1" (for version "d"), "hMBC1Le λ pCOS1" (for version "e") and "hMBC1Lf λ pCOS1" (for version "f").

[0251] Humanized L-chain versions "g" and "h" were also prepared using mutagenesis by PCR method. The versions "g" and "h" were designed such that the 36-position amino acid histidine (corresponding to the 36th amino acid in accordance with the Kabat's prescription) was replaced by tyrosine in the versions "a" and "d", respectively. The PCR reaction was performed using a mutagenic primer MBC1LGP9R (SEQ ID NO: 39), M13 Primer RV and plasmid hMBC1La λ pUC19 as a template. An additional PCR was performed using the PCR product thus obtained and M13 Primer M4 as primers and plasmid hMBC1La λ pUC19 as a template. The DNA fragment obtained was digested with HindIII and BlnI and then subcloned into plasmid C λ pUC19 that had been digested with HindIII and BlnI. Using this plasmid as a template, a PCR reaction was performed using primers MBC1LGP13R (SEQ ID NO: 40) and MBC1LVS1. The PCR fragment obtained was digested with ApaI and HindIII and then introduced into either of plasmids hMBC1La λ pUC19 and hMBC1Ld λ pUC19 that had been digested with ApaI and HindIII. The plasmids obtained were sequenced. Plasmids that were confirmed to contain the correct sequence were designated as "hMBC1Lg λ pUC19" (for version "g") and "hMBC1Lh λ pUC19" (for version "h"). Each of these plasmids was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmids thus obtained were respectively designated as "hMBC1Lg λ pCOS1" (for version "g") and "hMBC1Lh λ pCOS1" (for version "h").

EP 1 197 225 A1

- [0252] Humanized L-chain versions "i", "j", "k", "l", "m", "n" and "o" were also prepared using mutagenesis by PCR method. The PCR reaction was performed using plasmid hMBC1L λ /pUC19 as a template and a mutagenic primer MBC1LGP14S (SEQ ID NO: 41) and a primer V1RV (λ) (SEQ ID NO: 43). The resulting DNA fragment was digested with *Apal* and *BlnI* and then subcloned into plasmid hMBC1L λ /pUC19 that had been digested with *Apal* and *BlnI*.
- 5 The obtained plasmid was sequenced, and the clone into which the mutation for each version was introduced was selected. The thus obtained plasmid was designated as "hMBC1L λ /pUC 19 (x=i, j, k, l, m, n or o)". This plasmid was digested with *EcoRI* to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the *EcoRI* site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream of the EF1 α promoter. The thus obtained plasmid was designated as "hMBC1L λ /pCOS1" (x = i, j, k, l, m, n or o). The DNA sequences (including the corresponding amino acid sequences) of the versions "i", "j", "m" and "o" are shown in SEQ ID Nos: 67, 68, 69 and 70, respectively. The amino acid sequences of these versions are also shown in SEQ ID Nos: 48, 49, 50 and 51, respectively.
- 10 [0253] Humanized L-chain versions "p", "q", "r", "s" and "t" were designed such that the 87-position amino acid (tyrosine) was replaced by isoleucine in the versions "i", "j", "m", "l" and "o", respectively. These versions were prepared utilizing an *Aor51MI* restriction site on FR3 and replacing that site of each of the versions "i", "j", "m", "l" or "o" by that site of the version "h". That is, an *Aor51HI* restriction fragment (514 bp) containing CDR3, a part of FR3 and the entire FR4 were removed from an expression plasmid hMBC1L λ /pCOS1 (x = i, j, m, l or o). To the removed site, an *Aor51HI* restriction fragment (514 bp) in the expression plasmid hMBC1L λ /pCOS, which containing CDR3 and a part of FR3 and the entire FR4, was ligated, so that the 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine. The resulting plasmid was sequenced. A clone of each of the versions "i", "j", "m" "l" and "o" in which 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine was selected. These modified versions respectively corresponding to the versions "i", "j", "m" "l" and "o" were designated as versions "p", "q", "s", "r" and "t", respectively. The obtained plasmid was designated as "hMBC1L λ /pCOS1 (x = p, q, s, r or t). The DNA sequences (including the corresponding amino acids) of the versions "q", "r", "s" and "t" are shown in SEQ ID Nos: 71, 72, 73 and 74, respectively. The amino acid sequences of these versions are also shown in SEQ ID Nos: 52, 53, 54 and 55, respectively.
- 20 [0254] Plasmid hMBC1L λ /pCOS1 was digested with *HindIII* and *EcoRI* and then subcloned into plasmid pUC19 that had been digested with *HindIII* and *EcoRI*. The plasmid thus obtained was designated as "hMBC1L λ /pUC19.
- 30 [0255] The positions of the replaced amino acids in the individual versions of the humanized L-chain are shown in Table 2 below.

Table 2

Versions	36	43	45	47	49	80	87
a							
b		P			D		
c						P	
d							I
e		P			D		I
f						P	I
g	Y						
h	Y						I
i	Y		K				
j	Y		K		D		
k	Y		K	V			
l	Y		K	V	D		
m	Y				D		
n	Y			V			
o	Y			V	D		
p	Y		K				I

EP 1 197 225 A1

Table 2 (continued)

Versions	36	43	45	47	49	80	87
q	Y		K		D		I
r	Y				D		I
s	Y		K	V	D		I
t	Y			V	D		I

In Table 2, capital letters represent the following amino acids: Y: tyrosine; P: proline; K: lysine; V: valine; D: aspartic acid; and I: isoleucine.

[0256] E. coli strains each containing plasmids hMBC1HcDNA/pUC19 and hMBC1Lq₁/pUC19 were designated as "Escherichia coli JM109 (hMBC1HcDNA/pUC19)" and "Escherichia coli JM109 (hMBC1Lq₁/pUC19)", respectively, which have been deposited under the terms of Budapest Treaty at the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Japan, (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on August 15, 1996, under the accession No. FERM BP-5629 for Escherichia coli JM109 (hMBC1HcDNA/pUC19), and FERM BP-5630 for Escherichia coli JM109 (hMBC1Lq₁/pUC19).

(5) Transfection into COS-7 cell

[0257] For the evaluation of the antigen-binding activity and the neutralizing activity of the hybrid antibodies and the humanized #23-57-137-1 antibodies, the above-prepared expression plasmids were expressed transiently in COS-7 cells. For the transient expression of the L-chain hybrid antibodies, each of the following combinations of plasmids were co-transfected into a COS-7 cell by electroporation using Gene Pulser (Bio Rad): hMBC1HcDNA/pCOS1 and h/mMBC1L(Δ)/neo; hMBC1HcDNA/pCOS1 and m/mMBC1LΔ/neo; hMBC1HcDNA/pCOS1 and m/mMBC1LΔ/neo; hMBC1HcDNA/pCOS1 and hmmMBC1L(Δ)/neo; and hMBC1HcDNA/pCOS1 and hmmMBC1L(Δ)/neo. That is, a cell suspension (0.8 ml) of COS-7 cells in PBS(-) (1x10⁷ cells/ml) was added with each combination of the plasmid DNAs (10 μg each). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μF. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in DMEM medium containing 2% Ultra Low IgG fetal calf serum (GIBCO), and then cultured using a 10-cm culture dish in a CO₂ incubator. After culturing for 72 hours, a culture supernatant was collected and centrifuged to remove cell debris. The solutions thus prepared were provided for use in the ELISA below.

[0258] For the transient expression of the humanized #23-57-137-1 antibodies, plasmids of hMBC1HcDNA/pCOS1 and hMBC1Lx₁/pCOS1 (x = a-l) were co-transfected into a COS-7 cell using Gene Pulser (Bio Rad) in the same manner as described for the above hybrid antibodies. The culture supernatants were prepared and provided for use in the ELISA below.

[0259] The purification of the hybrid antibodies and the humanized antibodies from the COS-7 cell culture supernatants was performed using AffiGel Protein A MAPSII kit (Bio Rad) in accordance with the instructions included in the kit.

(6) ELISA

(i) Determination of antibody concentration

[0260] An ELISA plate for determining antibody concentration was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μl of a coating buffer (0.1 M NaHCO₃, 0.02% Na₂S₂O₃ containing 1 μg/ml of goat anti-human IgG antibody (TAGO) and then blocked with 200 μl of a dilution buffer [50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, 0.05% Tween 20, 0.02% Na₂S₂O₃, 1% bovine serum albumin (BSA); pH 7.2]. Each of the wells was added with each of the serial dilutions of the COS cell culture supernatant in which each of the hybrid antibodies and the humanized antibodies was expressed, or added with each of the serial dilutions of each of the hybrid antibodies and humanized antibodies in a purified form. The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Subsequently, each of the wells was added with 100 μl of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO). The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Subsequently, each of the wells was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution in each well was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad) to determine the antibody concentration. In this determination, Hu IgG1A Purified (The Binding Site) was used as the standard substance.

EP 1 197 225 A1

(ii) Determination of antigen-binding ability

[0261] An ELISA plate for determining antigen-binding ability was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μ l of a coating buffer containing 1 μ g/ml of human PTHrP (1-34) and then blocked with 200 μ l of a dilution buffer. Subsequently, each well was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the hybrid antibodies and humanized antibodies was expressed, or added with each of the serial dilutions of each of the hybrid antibodies and humanized antibodies in a purified form. The plate was incubated at room temperature and washed with PBS-Tween 20. Subsequently, each well was added with 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO). The plate was incubated at room temperature and washed with PBS-Tween 20. Subsequently, each well was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad).

(7) Confirmation of activities

(i) Evaluation of humanized H-chain

[0262] It was found that an antibody having both a humanized H-chain version "a" and a chimeric L-chain exhibited the same level of PTHrP-binding activity as that of a chimeric antibody. This result suggests that the version "a" achieves the humanization of the H-chain V-region in the degree enough to evaluate the humanization. Therefore, the humanized H-chain version "a" was provided for use as a humanized antibody H-chain in the following experiments.

(ii) Activity of hybrid antibodies

(ii-a) FR1,2/FR3,4 hybrid antibody

[0263] When the L-chain was h/mMBC1L(λ), no antigen-binding activity was observed. In contrast, when the L-chain was either m/hMBC1L λ or mvhMBC1Ld λ , the same level of antigen-binding activity as that of the chimeric #23-57-137-1 antibody was observed (FIG. 7). These results suggest that FR3 and FR4 have no problem as humanized antibodies but FR1 and FR2 contain amino acid residue(s) that need to be replaced.

(ii-b) FR1/FR2 hybrid antibody

[0264] When the L-chain was h/mhMBC1L(λ), no antigen-binding activity was observed. In contrast, when the L-chain was h/mhMBC1L(λ), the same level of antigen-binding activity as that of the chimeric #23-57-137-1 antibody was observed (FIG. 8). These results suggest that FR1 has no problem as a humanized antibody but FR2 contains amino acid residue(s) that need to be replaced.

(iii) Activity of humanized antibodies

[0265] The antigen-binding activity of the humanized antibodies having the L-chain versions "a" to "f", respectively, were determined. As a result, it was found that the humanized antibodies having the L-chain versions "j", "f", "m", "o", "q", "r", "s" and "t" exhibited the same levels of PTHrP-binding activity as that of the chimeric antibody.

(8) Establishment of CHO cell line capable of stable production of antibody

[0266] For establishing a cell line capable of stable production of humanized antibodies, each of the above-prepared expression plasmids was introduced into a CHO cell (DXB11).

[0267] That is, the establishment of a cell line capable of stable production of a humanized antibody was performed using each of the following combinations of plasmids as expression vectors for a CHO cell; hMBC1HcDNA/pCHO1 and hMBC1Lm λ /pCOS1; hMBC1HcDNA/pCHO1 and hMBC1Lq λ /pCOS1; and hMBC1HcDNA/pCHO1 and hMBC1Lr λ /pCOS1. The plasmids were co-transfected into a CHO cell by electroporation using Gene Pulser (Bio Rad). Subsequently, the expression vectors were separately cleaved with restriction enzyme PvuII to give linear DNA fragments. The resulting DNA fragments were extracted with phenol and chloroform and then precipitated with ethanol. The DNA fragments thus prepared were used in the subsequent electroporation. That is, the plasmid DNA fragments (10 μ g each) were added to 0.8 ml of a cell suspension of CHO cells in PBS(-) (1×10^7 cells/ml). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μ F. After 10 min. of recovery period at room temperature, the cells thus treated were suspended in MEM- α medium (GIBCO) containing 10% fetal calf serum (GIBCO),

EP 1 197 225 A1

and then cultured in a CO₂ incubator using 96-well plates (Falcon). On the day following the culturing being started, the medium was replaced by ribonucleoside- or deoxyribonucleoside-free MEM- α selective medium containing 10% fetal calf serum (GIBCO) and 500 mg/ml of GENETICIN (G418Sulfate; GIBCO). From the culture medium, cells into which the antibody gene was introduced were selected. The culture medium was replaced by a fresh one. About two weeks after the medium replacement, the cells were observed microscopically. When a satisfactory cell growth was observed, the amount of the antibodies produced was determined by conventional ELISA for determination of antibody concentration as set forth above. Among the cells, those cells which produced a larger amount of antibodies were screened.

[0268] The culturing of the established cell line capable of stable production of antibodies was scaled up in a roller bottle using a ribonucleoside- or deoxyribonucleoside-free MEM- α medium containing 2% Ultra Low IgG fetal calf serum. On each of day 3 and day 4 of the culturing, the culture supernatant was collected and filtered on a 0.2- μ m filter (Millipore) to remove cell debris therefrom. The purification of the humanized antibodies from the culture supernatant of the CHO cells was performed using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with the appended instructions. The humanized antibodies were provided for use in the determination of neutralizing activity and examination of pharmacological efficacy in hypercalcemic model animals. The concentration and the antigen-binding activity of the purified humanized antibodies were determined by the ELISA system as set forth above.

[REFERENCE EXAMPLE 5] Determination of neutralizing activity

[0269] The determination of neutralizing activity of the mouse antibodies, the chimeric antibodies and the humanized antibodies was performed using rat myeloma cell line ROS17/2.8-5 cells. The ROS17/2.8-5 cells were cultured in Ham's F-12 medium (GIBCO) containing 10% fetal calf serum (GIBCO) in a CO₂ incubator. The ROS17/2.8-5 cells were seeded into each well of a 96-well plate at a density of 10⁴ cells/100 μ l/well and cultured for one day. After the culturing was completed, the culture medium was replaced by Ham's F-12 medium (GIBCO) containing 4 mM Hydrocortisone and 10% fetal calf serum. After culturing for three to four days, the cultured cells were washed with 260 μ l of Ham's F-12 medium (GIBCO), and then added with 80 μ l of Ham's F-12 medium containing 1 mM isobutyl-1-methyl xanthine (IBMX, SIGMA), 10% fetal calf serum and 10 mM HEPES. The resulting mixture was incubated at 37° C for 30 min.

[0270] The culture mediums of the mouse antibodies, the chimeric antibodies and the humanized antibodies to be tested for neutralizing activity were previously diluted serially in the following dilution series: [10 μ g/ml, 3.3 μ g/ml, 1.1 μ g/ml and 0.37 μ g/ml], [10 μ g/ml, 2 μ g/ml, 0.5 μ g/ml and 0.01 μ g/ml] and [10 μ g/ml, 5 μ g/ml, 1.25 μ g/ml, 0.63 μ g/ml and 0.31 μ g/ml]. Each of the diluted antibody sample solutions was mixed with an equivalent amount of 4 ng/ml of PTHrP (1-34). The resulting mixed solution (80 μ l) was added to each well. In each well, the final concentration of each antibody became a quarter of the above-mentioned concentration of the antibody, and accordingly the concentration of PTHrP (1-34) became 1 ng/ml. After the treatment at room temperature for 10 min., the culture supernatant was removed and the residue was washed with PBS three times. Subsequently, cAMP in the cells was extracted with 100 μ l of a 0.3% HCl-95% ethanol and then evaporated using a water jet aspirator to remove the HCl-ethanol. The residue was dissolved in 120 μ l of EIA buffer appended to cAMP EIA Kit (CAYMAN CHEMICAL'S) to extract the cAMP therefrom. The cAMP was determined using cAMP EIA Kit (CAYMAN CHEMICAL'S) in accordance with the instructions included in the kit. As a result, it was found that, among the humanized antibodies having the same levels of antigen-binding activity as that of the chimeric antibody, those antibodies having L-chain versions "q", "r", "s" and "t" (in which the 91-position tyrosine was replaced by isoleucine) exhibited the similar neutralizing activity to that of the chimeric antibody, and that antibody having a L-chain version "q" exhibited the strongest neutralizing activity.

Sequence Listing Free Text

[0271]

SEQ ID NO: 1 Synthesized DNA
 SEQ ID NO: 2 Synthesized DNA
 SEQ ID NO: 3 Synthesized DNA
 SEQ ID NO: 4 Synthesized DNA
 SEQ ID NO: 5 Synthesized DNA
 SEQ ID NO: 6 Synthesized DNA
 SEQ ID NO: 7 Synthesized DNA
 SEQ ID NO: 8 Synthesized DNA
 SEQ ID NO: 9 Synthesized DNA

EP 1 197 225 A1

SEQ ID NO: 10 Synthesized DNA
 SEQ ID NO: 11 Synthesized DNA
 SEQ ID NO: 12 Synthesized DNA
 SEQ ID NO: 13 Synthesized DNA
 5 SEQ ID NO: 14 Synthesized DNA
 SEQ ID NO: 15 Synthesized DNA
 SEQ ID NO: 16 Synthesized DNA
 SEQ ID NO: 17 Synthesized DNA
 10 SEQ ID NO: 18 Synthesized DNA
 SEQ ID NO: 19 Synthesized DNA
 SEQ ID NO: 20 Synthesized DNA
 SEQ ID NO: 21 Synthesized DNA
 SEQ ID NO: 22 Synthesized DNA
 15 SEQ ID NO: 23 Synthesized DNA
 SEQ ID NO: 24 Synthesized DNA
 SEQ ID NO: 25 Synthesized DNA
 SEQ ID NO: 26 Synthesized DNA
 SEQ ID NO: 27 Synthesized DNA
 20 SEQ ID NO: 28 Synthesized DNA
 SEQ ID NO: 29 Synthesized DNA
 SEQ ID NO: 30 Synthesized DNA
 SEQ ID NO: 31 Synthesized DNA
 SEQ ID NO: 32 Synthesized DNA
 25 SEQ ID NO: 33 Synthesized DNA
 SEQ ID NO: 34 Synthesized DNA
 SEQ ID NO: 35 Synthesized DNA
 SEQ ID NO: 36 Synthesized DNA
 SEQ ID NO: 37 Synthesized DNA
 30 SEQ ID NO: 38 Synthesized DNA
 SEQ ID NO: 39 Synthesized DNA
 SEQ ID NO: 40 Synthesized DNA
 SEQ ID NO: 41 Synthesized DNA
 SEQ ID NO: 42 Synthesized DNA
 35 SEQ ID NO: 43 Synthesized DNA
 SEQ ID NO: 44 Synthesized DNA

[0272] All publications, patents and patent applications cited herein are incorporated by reference in their entirety.

INDUSTRIAL APPLICABILITY

[0273] The present invention provides a therapeutic agent for diseases caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.

EP 1 197 225 A1

SEQUENCE LISTING

5

<110> CHUGAI SEIYAKU KABUSHIKI KAISHA

10

<120> Therapeutic agent for diseases caused with PTH or PTHrP

15

<130> PH-945-PCT

20

<150> JP 11-189793

<151> 1999-07-02

25

<160> 75

<170> PatentIn Ver. 2.0

30

<210> 1

<211> 20

35

<212> DNA

<213> Artificial Sequence

40

<220>

<223> Synthetic DNA

45

<400> 1

aaatagccct tgaccaggca

20

50

<210> 2

<211> 38

55

<212> DNA

EP 1 197 225 A1

5 <213> Artificial Sequence

 <220>

 <223> Synthetic DNA

10

 <400> 2

15 ctggctcggc ccacctctga aggttcaga atcgatag 38

 <210> 3

20 <211> 28

 <212> DNA

 <213> Artificial Sequence

25

 <220>

 <223> Synthetic DNA

30

 <400> 3

35 ggatcccggg ccagtgata gacagatg 28

 <210> 4

40 <211> 29

 <212> DNA

 <213> Artificial Sequence

45

 <220>

50 <223> Synthetic DNA

 <400> 4

55 ggatcccggg tcagrgaag gtggraaca 29

EP 1 197 225 A1

5 <210> 5
 <211> 17
 <212> DNA
10 <213> Artificial Sequence

15 <220>
 <223> Synthetic DNA

20 <400> 5
gttttccag tcacgac 17

25 <210> 6
 <211> 17
 <212> DNA
30 <213> Artificial Sequence

35 <220>
 <223> Synthetic DNA

40 <400> 6
caggaaacag ctatgac 17

50

$\langle 210 \rangle$	7
$\langle 211 \rangle$	31
$\langle 212 \rangle$	DNA
$\langle 213 \rangle$	Artificial Sequence

55 $\langle 220 \rangle$

EP 1 197 225 A1

<223> Synthetic DNA

5

<400> 7

gictaagctt ocaccaigaa acttcgggct c 31

10

<210> 8

<211> 30

15

<212> DNA

<213> Artificial Sequence

20

<220>

<223> Synthetic DNA

25

<400> 8

tgttggatcc ctgcagagac agtgaccaga 30

30

<210> 9

<211> 36

35

<212> DNA

<213> Artificial Sequence

40

<220>

<223> Synthetic DNA

45

<400> 9

gctcgaattc aagcttcac calggggttt gggctg 36

50

<210> 10

55

<211> 41

EP 1 197 225 A1

<212> DNA
 5 <213> Artificial Sequence

 <220>
 10 <223> Synthetic DNA

 <400> 10
 15 ttccccgggc ccttgggga ggctgaggag acggtgacca g 41

 <210> 11
 20 <211> 109
 <212> DNA
 25 <213> Artificial Sequence

 <220>
 30 <223> Synthetic DNA

 <400> 11
 35 gtcigaattc aagcttagta ctggccagc ccaaggccaa cccacgggc accctgttc 60
 40 cggcctcttc tgaggagtc caagccaaca aggcacact agtgtgtc 109

 <210> 12
 45 <211> 110
 <212> DNA
 <213> Artificial Sequence
 50
 <220>
 55 <223> Synthetic DNA

EP 1 197 225 A1

<400> 12
 5 gggttgggtggtg tctccactcc cgccttgacg gggctggcat ctgccttcca ggccactgic 60
 acagctcccg ggtagaatic actgacaga cacactagtg tggccttggt 110

10 <210> 13
 <211> 98
 <212> DNA
 15 <213> Artificial Sequence

20 <220>
 <223> Synthetic DNA
 25 <400> 13
 ggagtgagga ccaccaaac ctccaacag agcaacaaca agtaccggc cagcagctac 60
 ctgagcciga cggccagca ggggaagtc cacagaag 98

30 <210> 14
 <211> 106
 <212> DNA
 <213> Artificial Sequence

40 <220>
 <223> Synthetic DNA

45 <400> 14
 50 tgttgaatic ttactatga caicctgtg gggccactgt ctctccacg gtgtccctt 60
 catcgctgac ctggcagctg tagcttcigt gggacttcca ctgctc 106

55 <210> 15

EP 1 197 225 A1

5 <211> 43
 <212> DNA
 <213> Artificial Sequence

10 <220>
 <223> Synthetic DNA

15 <400> 15
 gtctgaattc aagcttagta ctggccagc ccaaggccaa ccc 43

20 <210> 16
 <211> 20
25 <212> DNA
 <213> Artificial Sequence

30 <220>
 <223> Synthetic DNA

35 <400> 16
 tgttgaattc ttactatgaa 20

40 <210> 17
 <211> 39
45 <212> DNA
 <213> Artificial Sequence

50 <220>
 <223> Synthetic DNA

55

EP 1 197 225 A1

<400> 17
 5 caacaagttac ggcgccagca gttacctgag cctgacgcc 39

<210> 18
 10 <211> 39
 <212> DNA
 15 <213> Artificial Sequence

<220>
 20 <223> Synthetic DNA

<400> 18
 25 gtactgctcg gccgcgtact tgttgttct ctttttga 39

<210> 19
 30 <211> 46
 <212> DNA
 35 <213> Artificial Sequence

<220>
 40 <223> Synthetic DNA

<400> 19
 45 gtcagaattc aagcttagtc ctaggctgaa ctgtggctgc accatc 46

<210> 20
 50 <211> 34
 <212> DNA
 55 <213> Artificial Sequence

EP 1 197 225 A1

5	<220>	
	<223> Synthetic DNA	
10	<400> 20	
	tgttgaattc ttactaacac tctccctcgt tgaa	34
15	<210> 21	
	<211> 35	
20	<212> DNA	
	<213> Artificial Sequence	
25	<220>	
	<223> Synthetic DNA	
30	<400> 21	
	gtctaagctt ccaccatggc ctggactcct ctctt	35
35	<210> 22	
	<211> 48	
40	<212> DNA	
	<213> Artificial Sequence	
45	<220>	
	<223> Synthetic DNA	
50	<400> 22	
	tgttgaattc agatctaact acttacctag gacagtgacc ttggctccc	48
55		

EP 1 197 225 A1

<210> 23

<211> 128

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 23

gtctaagctt ccacatggg gttgggctg agcigggtt tctcgttgc tctttaaga 60
gggtccagt gtcaggta gctggggag tctggggag gcgtgtcca gctgggag 120
tccc:gag 128

<210> 24

<211> 125

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic DNA

<400> 24

accattagta gtgtgtag ttacacctac tatccagaca ggtgaaggc gcgattacc 60
atctccagag acaattccaa gaacacgctg tatctgcaa tgaacagcct gagagctgag 120
gacac 125

<210> 25

<211> 132

<212> DNA

EP 1 197 225 A1

<213> Artificial Sequence

5

<220>

<223> Synthetic DNA

10

<400> 25

15

ctaccaccac tactaatggt tgcacccac tccagccct tgcctggagc ctggcggacc 60
caagacatgc catagtact gaaggtaga ctagagctg cacaggagag tctcaggac 120
ctccaggct gg 132

20

<210> 26

<211> 110

25

<212> DNA

<213> Artificial Sequence

30

<220>

<223> Synthetic DNA

35

<400> 26

40

tgttggatcc ctgaggagc ggtgaccagg gtccctggc cccagtaagc aaagtaagtc 60
atagtagtct gtctgcaca gtaatacaca gcgtgtcct cagctctcag 110

45

<210> 27

<211> 30

<212> DNA

50

<213> Artificial Sequence

55

<220>

<223> Synthetic DNA

EP 1 197 225 A1

5	<400> 27	
	gtctaagctt ccaccatggg gtttggctg	30
10	<210> 28	
	<211> 30	
	<212> DNA	
15	<213> Artificial Sequence	
20	<220>	
	<223> Synthetic DNA	
25	<400> 28	
	tgttgatcc ctgaggagac ggtgaccag	30
30	<210> 29	
	<211> 133	
35	<212> DNA	
	<213> Artificial Sequence	
40	<220>	
	<223> Synthetic DNA	
45	<400> 29	
	acaaagcttc caccatggcc tggactctc tctttctct cttgttctt catggctcag 60	
50	gtttcttct ccagcttctg ctgactcaat cgcctctgc ctctgctcc ctggagctt 120	
	cggtaagct cac	133
55	<210> 30	

EP 1 197 225 A1

5
10
15
20
25
30
35
40
45
50
55

<211> 118
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic DNA

<400> 30
agcaagatgg agccacagc acaggtgatg ggaatcctga tgccttcctca ggcctccagct 60
ctgggctga ggcctaccct accatctcca gccctccagtc tgaggatgag gctgacta 118

<210> 31
<211> 128
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic DNA

<400> 31
ctgtggcttc catcttgctt aagtttcac aagtaccgag ggcccttcctc tgcctgctgc 60
tgatgccatt caatgggta cgtactgtgc tgactactca aggtccaggt gacgttgacc 120
gaggctcc 128

<210> 32
<211> 114
<212> DNA
<213> Artificial Sequence

EP 1 197 225 A1

<220>

5 <223> Synthetic DNA

<400> 32

10 cttggatccg ggctgacctt ggacggctcag ttgggtccct ccgccgaaca ccttcacaaa 60
ttgttcctta attgtatcac ccacaccaca gtaatagta gccctatcct caga 114

15 <210> 33

<211> 17

20 <212> DNA

<213> Artificial Sequence

25 <220>

<223> Synthetic DNA

30 <400> 33

acaaagcttc caccatg 17

35 <210> 34

<211> 19

40 <212> DNA

<213> Artificial Sequence

45 <220>

<223> Synthetic DNA

50 <400> 34

cttggatccg ggctgacctt 19

55

EP 1 197 225 A1

```

5      <210> 35
      <211> 75
      <212> DNA
      <213> Artificial Sequence

10

      <220>
      <223> Synthetic DNA

15

      <400> 35
20      ctggatcgc ggcgacctt ggacgctcag ttgtgtccct ccgccgaaca cgtacacaa 60
      ttgttccta attgt 75

25

      <210> 36
      <211> 43
      <212> DNA
30      <213> Artificial Sequence

      .
35      <220>
      <223> Synthetic DNA

40

      <400> 36
      aaagatcct taagatcat caagtaccga gggggcttct ctg 43

45

      <210> 37
      <211> 46
      <212> DNA
50      <213> Artificial Sequence

55

      <220>

```

EP 1 197 225 A1

<223> Synthetic DNA

5

<400> 37

acaaagctta ggcctaccic accatctcca gccctcagcc tgagga 46

10

<210> 38

<211> 111

15

<212> DNA

<213> Artificial Sequence

20

<220>

<223> Synthetic DNA

25

<400> 38

cttggatcgg ggcctgacctt ggacggctag ttctgtccct ccgcgaaca cgtacacaa 60

30

ttgttcttta attgtatcac ccacaccaca gatatagtca ggcctatcct c 111

35

<210> 39

<211> 42

<212> DNA

40

<213> Artificial Sequence

<220>

45

<223> Synthetic DNA

<400> 39

50

ctttctcggc tgctgctgat accattcaat gggtctagta ct 42

55

<210> 40

EP 1 197 225 A1

	<211> 26	
5	<212> DNA	
	<213> Artificial Sequence	
10	<220>	
	<223> Synthetic DNA	
15	<400> 40	
	cgaggggccct tctctggc:g ctgc:tg	26
20	<210> 41	
	<211> 35	
25	<212> DNA	
	<213> Artificial Sequence	
30	<220>	
	<223> Synthetic DNA	
35	<400> 41	
	gagaaggccc ctargtacst gatgrawctt aagca	35
40	<210> 42	
	<211> 35	
45	<212> DNA	
	<213> Artificial Sequence	
50	<220>	
	<223> Synthetic DNA	
55		

EP 1 197 225 A1

<400> 42
 5 caccgaattca ctatcgattc tggaaacctc agagg 35

 <210> 43
 10 <211> 18
 <212> DNA
 15 <213> Artificial Sequence

 <220>
 20 <223> Synthetic DNA

 <400> 43
 25 ggcttggagc tcctcaga 18

 <210> 44
 30 <211> 20
 <212> DNA
 35 <213> Artificial Sequence

 <220>
 40 <223> Synthetic DNA

 <400> 44
 45 gacagtgggtt caaagttttt 20

 <210> 45
 <211> 118
 <212> PRT
 55 <213> Mus musculus

EP 1 197 225 A1

5 <400> 45
 Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser Leu Gly Ala
 1 5 10 15
 10 Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 15 Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys Tyr Val Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 20 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu Ser Ile Ser
 65 70 75 80
 25 Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly Val Gly Asp
 85 90 95
 30 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Val
 100 105 110
 Thr Val Leu Gly Gln Pro
 35 115

40 <210> 46
 <211> 118
 <212> PRT
 45 <213> Mus musculus

50 <400> 46
 Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys Pro Gly Gly
 1 5 10 15
 55 Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
 20 25 30

EP 1 197 225 A1

Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp Val
 35 40 45
 5 Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
 50 55 60
 10 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80
 15 Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met Phe Tyr Cys
 85 90 95
 Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110
 20 Leu Val Thr Val Ser Ala
 115

25

<210> 47

<211> 116

30

<212> PRT

<213> Homo sapiens

35

<400> 47

40

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 45 Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 35 40 45
 50 Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 55 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80

EP 1 197 225 A1

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly
 115
 <210> 48
 <211> 118
 <212> PRT
 <213> Homo sapiens
 <400> 48
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly Gln Pro

EP 1 197 225 A1

115

5

<210> 49

<211> 118

10

<212> PRT

<213> Homo sapiens

15

<400> 49

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala

20

1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr

20 25 30

25

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met

35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp

30

50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser

35

65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp

85 90 95

40

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu

100 105 110

Thr Val Leu Gly Gln Pro

45

115

50

<210> 50

<211> 118

<212> PRT

55

<213> Homo sapiens

EP 1 197 225 A1

5 <400> 50
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 10 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 15 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 20 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 25 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 30 100 105 110
 Thr Val Leu Gly Gln Pro
 35 115

40 <210> 51
 <211> 118
 <212> PRT
 45 <213> Homo sapiens

50 <400> 51
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 55 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30

EP 1 197 225 A1

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly Gln Pro
 115

<210> 52
 <211> 118
 <212> PRT
 <213> Homo sapiens

<400> 52
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80

EP 1 197 225 A1

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 5 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 10 Thr Val Leu Gly Gln Pro
 115
 15
 <210> 53
 <211> 118
 20 <212> PRT
 <213> Homo sapiens
 25
 <400> 53
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 30 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 35 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 40 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 45 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85 90 95
 50 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly Gln Pro
 55 115

EP 1 197 225 A1

5 <210> 54
 <211> 118
 <212> PRT
 10 <213> Homo sapiens

15 <400> 54
 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 20 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
 25 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 30 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 35 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 40 100 105 110
 Thr Val Leu Gly Gln Pro
 115

45 <210> 55
 50 <211> 118
 <212> PRT
 <213> Homo sapiens
 55

EP 1 197 225 A1

<400> 55

5 Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 10 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
 35 40 45
 15 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 20 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 25 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 30 Thr Val Leu Gly Gln Pro
 115

<210> 56

<211> 118

<212> PRT

<213> Homo sapiens

<400> 56

50 Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
 20 25 30
 55 Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val

EP 1 197 225 A1

35 40 45
 5 Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 10 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 15 Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110
 20 Leu Val Thr Val Ser Ser
 115

 25 <210> 57
 <211> 411
 <212> DNA
 30 <213> Mus musculus

 35 <220>
 <221> CDS
 <222> (1).. (411)
 40
 <220>
 <221> mat_peptide
 45 <222> (58).. (411)

 50 <400> 57
 atg aac ttc ggg etc agc ttc att ttc ctt gcc etc att tta aaa ggt 48
 Met Asn Phe Gly Leu Ser Leu Ile Phe Leu Ala Leu Ile Leu Lys Gly
 55 -15 -10 -5

EP 1 197 225 A1

gtc cag tgt gag gtg caa ctg gtg gag tct ggg gga gac tta gtg aag 96
 5 Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys
 -1 1 5 10
 cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc 144
 10 Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 15 20 25
 agt agc tat ggc atg tct tgg att cgc cag act cca gac aag agg ctg 192
 15 Ser Ser Tyr Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu
 30 35 40 45
 gag tgg gtc gca acc att agt agt ggt agt tac acc tac tat cca 240
 20 Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
 50 55 60
 gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat gcc aag aac 288
 25 Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
 65 70 75
 acc cta tac ctg caa atg agc agt ctg aag tct gag gac aca gcc atg 336
 30 Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met
 80 85 90
 ttt tac tgt gca aga cag act act atg act tac ttt gct tac tgg ggc 384
 35 Phe Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly
 95 100 105
 caa ggg act ctg gtc act gtc tct gca 411
 40 Gln Gly Thr Leu Val Thr Val Ser Ala
 45 110 115
 50 <210> 58
 <211> 411
 <212> DNA
 55 <213> Homo sapiens

EP 1 197 225 A1

5 <220>
 <221> CDS
 <222> (1).. (411)

10
 <220>
 <221> mal_peptide
 15 <222> (58).. (411)

20 <400> 58
 atg ggg ttt ggg ctg agc tgg gtt ttc ctc gtt gct ctt tta aga ggt 48
 Met Gly Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
 25 -15 -10 -5
 gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc gtg gtc cag 96
 Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Val Val Gln
 30 -1 1 5 10
 cct ggg agg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc 144
 35 Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 15 20 25
 agt agc tat ggc atg tct tgg gtc cgc cag gct cca ggc aag ggg ctg 192
 40 Ser Ser Tyr Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 30 35 40 45
 gag tgg gtg gca acc att agt agt ggt ggt agt tac acc tac tat cca 240
 45 Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
 50 55 60
 gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat tcc aag aac 288
 50 Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
 65 70 75
 55 acg ctg tat ctg caa atg aac agc ctg aga gct gag gac acg gct gtc 336

EP 1 197 225 A1

Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
 80 85 90
 5 tat tac tgt gcg aga cag act act atg act tac ttt gct tac tgg ggc 384
 Tyr Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly
 10 95 100 105
 cag gga acc ctg gtc acc gtc tcc tca 411
 Gln Gly Thr Leu Val Thr Val Ser Ser
 15 110 115
 20 <210> 59
 <211> 11
 <212> PRT
 25 <213> Homo sapiens
 <400> 59
 30 Lys Ala Ser Gln Asp Val Asn Thr Ala Val Ala
 1 5 10
 35 <210> 60
 <211> 7
 40 <212> PRT
 <213> Homo sapiens
 45 <400> 60
 Ser Ala Ser Asn Arg Tyr Thr
 50 1 5
 <210> 61
 55 <211> 9

EP 1 197 225 A1

<212> PRT
 <213> Homo sapiens
 5

 <400> 61
 10 Gln Gln His Tyr Ser Thr Pro Phe Thr
 1 5

 15 <210> 62
 <211> 5
 20 <212> PRT
 <213> Homo sapiens

 25 <400> 62
 Pro Tyr Trp Met Gln
 1 5
 30

 <210> 63
 35 <211> 16
 <212> PRT
 <213> Homo sapiens
 40

 <400> 63
 45 Ser Ile Phe Gly Asp Gly Asp Thr Arg Tyr Ser Gln Lys Phe Lys Gly
 1 5 10 15

 50 <210> 64
 <211> 11
 <212> PRT
 55 <213> Homo sapiens

EP 1 197 225 A1

5 <400> 64
 Gly Leu Arg Arg Gly Gly Tyr Tyr Phe Asp Tyr
 1 5 10
 10
 <210> 65
 <211> 411
 15 <212> DNA
 <213> Mus musculus
 20
 <220>
 <221> CDS
 25 <222> (1).. (411)
 30
 <220>
 <221> mat_peptide
 <222> (58).. (411)
 35
 <400> 65
 atg gcc tgg act cct ctc ttc ttc ttc tit gtt ctt cat tgc tca ggt 48
 40 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5
 tct ttc tcc caa ctt gtg ctc act cag tca tct tca gcc tct ttc tcc 96
 45 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser
 -1 1 5 10
 50 ctg gga gcc tca gca aaa ctc acg tgc acc ttg agt agt cag cac agt 144
 Leu Gly Ala Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 55 acg tac acc att gaa tgg tat cag caa cag cca ctc aag cct cct aag 192

EP 1 197 225 A1

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys
 30 35 40 45
 tat gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 att cct gat cgc ttc tct gga tcc agc tct ggt gct gat cgc tac ctt 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu
 65 70 75
 agc att tcc aac atc cag cca gaa gat gaa gca atg tac atc tgt ggt 336
 Ser Ile Ser Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly
 80 85 90
 gtg ggt gat aca att aag gaa caa ttt gtg tat gtt ttc ggc ggt ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105
 acc aag gtc act gtc cta ggt cag ccc 411
 Thr Lys Val Thr Val Leu Gly Gln Pro
 110 115
 <210> 66
 <211> 411
 <212> DNA
 <213> Homo sapiens
 <220>
 <221> CDS
 <222> (1).. (411)
 <220>
 <221> mat_peptide

EP 1 197 225 A1

<222> (58).. (411)

5

<400> 66

10

atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly

-15

-10

-5

15

tct ttc tcc cag ctt gtg ctg act caa tgc ccc tct gcc tct gcc tcc 96

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser

-1

1

5

10

20

ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

15

20

25

25

acg tac acc att gaa tgg cat cag cag cag cca gag aag ggc cct cgg 192

Thr Tyr Thr Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg

30

35

40

45

30

tac ttg atg aaa ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Leu Met Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

35

50

55

60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

40

65

70

75

acc aic tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly

45

80

85

90

gtg ggt gat aca att aag gaa caa tit gtg tac gtg ttc ggc gga ggg 384

50

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95

100

105

acc aaa ctg acc gtc cta ggt cag ccc 411

55

Thr Lys Leu Thr Val Leu Gly Gln Pro

EP 1 197 225 A1

110 115

5 <210> 67
 <211> 411
 10 <212> DNA
 <213> Homo sapiens

15 <220>
 <221> CDS
 20 <222> (1).. (411)

<220>
 25 <221> mat_peptide
 <222> (58).. (411)

30 <400> 67
 atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 35 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5
 tct ttc tcc cag ctt gtg ctg act caa tgc ccc tct gcc tct gcc tcc 96
 40 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 -1 1 5 10
 ctg gga gcc tgg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
 45 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 50 acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
 30 35 40 45
 55 tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

EP 1 197 225 A1

Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 5 att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75
 10 acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
 80 85 90
 15 gtg ggt gat aca att aag gaa caa tit gtg tac gtg ttc ggc gga ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105
 20 acc aaa ctg acc gtc cta ggc cag ccc 411
 25 Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115
 30 <210> 68
 <211> 411
 35 <212> DNA
 <213> Homo sapiens
 40 <220>
 <221> CDS
 <222> (1).. (411)
 45
 <220>
 50 <221> mat_peptide
 <222> (58).. (411)
 55 <400> 68

EP 1 197 225 A1

5 atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5
 10 ttt ttc tcc cag ctt gtg ctg act caa tgc ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 -1 1 5 10
 15 ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 20 acc tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
 30 35 40 45
 25 tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 30 att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75
 35 acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
 80 85 90
 40 gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105
 45 acc aaa ctg acc gtc cta ggc cag ccc 411
 Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115
 55 <210> 69

EP 1 197 225 A1

<21> 411
 <212> DNA
 <213> Homo sapiens

 <220>
 <221> CDS
 <222> (1).. (411)

 <220>
 <221> mat_peptide
 <222> (53).. (411)

 <400> 69
 atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5
 tct ttc tcc cag ctt gtg ctg act caa tgg ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 -1 1 5 10
 ctg gga gcc tgg gtc aag ctc acc tgc acc ttt agt agt cag cac agt 144
 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
 30 35 40 45
 tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

EP 1 197 225 A1

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 5 65 70 75
 acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
 10 80 85 90
 gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 15 95 100 105
 acc aaa ctg acc gtc cta ggc cag ccc 411
 Thr Lys Leu Thr Val Leu Gly Gln Pro
 20 110 115

 25 <210> 70
 <211> 411
 30 <212> DNA
 <213> Homo sapiens

 35 <220>
 <221> CDS
 <222> (1).. (411)
 40
 <220>
 <221> mat_peptide
 45 <222> (58).. (411)

 50 <400> 70
 atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Val Leu His Cys Ser Gly
 55 -15 -10 -5

EP 1 197 225 A1

tct ttc tcc cag ctt gig ctg act caa tgg ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 5 -1 1 5 10
 ctg gga gcc tgg gtc aag ctc acc tgc acc tgg agt agt cag cac agt. 144
 10 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192
 15 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
 30 35 40 45
 20 tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 25 att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75
 30 acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
 80 85 90
 35 gtg ggt gat aca att aag gaa caa ttt gtg tac gig ttc ggc gga ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 40 95 100 105
 acc aaa ctg acc gtc cta ggc cag ccc 411
 45 Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115
 50 <210> 71
 <211> 411
 <212> DNA
 55 <213> Homo sapiens

EP 1 197 225 A1

5 <220>
 <221> CDS
 <222> (1).. (411)
 10
 <220>
 <221> mat_peptide
 15 <222> (58).. (411)
 20 <400> 71
 alg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 25 -15 -10 -5
 tct ttc tcc cag ctt gig ctg acf caa tgc ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 30 -1 1 5 10
 ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 35 15 20 25
 acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
 40 30 35 40 45
 tac ctg alg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 45 50 55 60
 att cct gat cgc ttc tca gcc tcc agc tct ggg gct gag cgc tac ctc 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 50 65 70 75
 55 acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336

EP 1 197 225 A1

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
 5 80 85 90
 gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 10 95 100 105
 acc aaa ctg acc gtc cta ggc cag ccc 411
 15 Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115

 20 <210> 72
 <211> 411
 <212> DNA
 25 <213> Homo sapiens

 30 <220>
 <221> CDS
 <222> (1).. (411)
 35
 <220>
 <221> mat_peptide
 40 <222> (58).. (411)

 45 <400> 72
 aig gcc tgg act cct ctc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 50 -15 -10 -5
 tct ttc tcc cag ctt gtg ctg act caa tgg ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 55 -1 1 5 10

EP 1 197 225 A1

5 c t g g g a g c c t c g g t c a a g c t c a c c t g c a c c t t g a g t a g t c a g c a c a g t 144
 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25
 10 a c g t a c a c c a t t g a a t g g t a t c a g c a g c a g c c a g a g a g g c c t a g g 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
 30 35 40 45
 15 t a c c t g a t g a t c i t a a g c a a g a t g g a a g c c a c a g c a c a g g t g a t g g g 240
 Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 20 a t t c c t g a t c g c t t c a g g c t c c a g c t c t g g g g c t g a g c g c t a c c t c 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75
 25 a c c a t c t c c a g c c t c a g t c t g a g g a t g a g g c t g a c t a t a t c t g t g g t 336
 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
 80 85 90
 30 g t g g g t g a t a c a a t t a a g g a a c a a t t t g t g a c g t g t t c g g c g g a g g g 384
 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105
 35 a c c a a c t g a c c g t c t a g g c c a g c c c 411
 Thr Lys Leu Thr Val Leu Gly Gln Pro
 40 110 115

45 <210> 73
 <211> 411
 <212> DNA
 50 <213> Homo sapiens

55 <220>
 <221> CDS

EP 1 197 225 A1

<222> (1).. (411)

5

<220>

<221> mat_peptide

10

<222> (58).. (411)

15

<400> 73

atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly

20

-15

-10

-5

tct ttc tcc cag ctt gtc ctg act caa tgc ccc tct gcc tct gcc tcc 96

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser

25

-1

1

5

10

ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

30

15

20

25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys

35

30

35

40

45

tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

40

50

55

60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

45

65

70

75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly

50

80

85

90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384

55

EP 1 197 225 A1

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 5 95 100 105
 acc aaa ctg acc gtc cta ggc cag ccc 411
 Thr Lys Leu Thr Val Leu Gly Gln Pro
 10 110 115

 15 <210> 74
 <211> 411
 <212> DNA
 20 <213> Homo sapiens

 <220>
 25 <221> CDS
 <222> (1).. (411)

 30 <220>
 <221> mat_peptide
 35 <222> (58).. (411)

 <400> 74
 40 atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5
 45 tct ttc tcc cag ctt gtc ctg act caa tgc ccc tct gcc tct gcc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 50 -1 1 5 10
 ctg gga gcc tgc gtc aag ctc acc tgc acc ttc agt agt cag cac agt 144
 Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 55 15 20 25

EP 1 197 225 A1

acg tac acc att gaa tgg tai cag cag cag cca gag aag ggc cct agg 192
 5 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
 30 35 40 45
 tac gtg alg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
 10 Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60
 att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac etc 288
 15 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75
 acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336
 20 Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
 80 85 90
 gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gaa ggg 384
 25 Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105
 acc aaa ctg acc gtc cta ggc cag ccc 411
 30 Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115
 35
 <210> 75
 <211> 34
 40 <212> FRT
 <213> Homo sapiens
 45
 <400> 75
 Ala Val Ser Glu His Gln Leu Leu His Asp Lys Gly Lys Ser Ile Gln
 50 1 5 10 15
 Asp Leu Arg Arg Arg Phe Phe Leu His His Leu Ile Ala Glu Ile His
 20 25 30
 55 Thr Ala

EP 1 197 225 A1

5

Claims

- 10 1. A therapeutic agent for a disease caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 15 2. The therapeutic agent according to claim 1, wherein the disease caused by PTH or PTHrP is primarily a disease other than hypercalcemia.
3. A QOL improving agent for alleviating a symptom of a disease caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 20 4. A therapeutic agent for a syndrome associated with malignancy caused by PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 25 5. The therapeutic agent according to claim 4, wherein the syndrome associated with malignancy is selected from the group consisting of digestive system disorders, proteometabolism abnormality, saccharometabolism abnormality, lipid metabolism abnormality, anorexia, hematological abnormality, electrolyte abnormality, immunodeficiency and pain.
- 30 6. The therapeutic agent according to claim 1 or 2, wherein the disease is a secondary hyperparathyroidism or primary hyperparathyroidism caused by PTH.
7. An alleviation agent for a central nervous system disease caused by PTH or PTHrP, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 35 8. The alleviation agent according to claim 7, wherein the central nervous system disease is selected from the group consisting of dyssomnia, neuropathy, nervous symptom, brain metabolism abnormality, cerebral circulation abnormality, autonomic imbalance, and endocrine system abnormality with which central nervous system is associated.
- 40 9. An alleviation agent for a disease caused by PTH or PTHrP-cytokine cascade, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 45 10. The alleviation agent according to claim 9, wherein the cytokine is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, G-CSF, GM-CSF, M-CSF, EPO, LIF, TPO, EGF, TGF- α , TGF- β , FGF, IGF, HGF, VEGF, NGF, activin, inhibin, a BMP family, TNF and IFN.
- 50 11. The alleviation agent according to claim 9 or 10, wherein the disease caused by PTH or PTHrP-cytokine cascade is selected from the group consisting of septicemia, cachexia, inflammation, hemopathy, calcium metabolism abnormality, and autoimmune disease.
12. A central nervous system regulator, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between the ligand and the receptor.
- 55 13. A cytokine network regulator, which comprises, as an active ingredient, an agonist or antagonist binding to a PTH receptor or PTHrP receptor, or a substance binding to a ligand of the receptor to promote or inhibit binding between

EP 1 197 225 A1

the ligand and the receptor.

14. The agent according to any one of claims 1 to 13, wherein the PTH receptor or PTHrP receptor is a PTH/PTHrP type I receptor.

15. The agent according to any one of claims 1 to 14, wherein the substance binding to a ligand of PTH receptor or PTHrP receptor to inhibit binding between the ligand and the receptor is selected from the group consisting of an anti-PTHrP antibody and anti-PTH antibody.

16. The agent according to claim 15, wherein the substance binding to a ligand of PTH receptor or PTHrP receptor to inhibit binding between the ligand and the receptor is an anti-PTHrP antibody.

17. The agent according to claim 16, wherein the anti-PTHrP antibody is a humanized anti-PTHrP antibody.

EP 1 197 225 A1

FIG. 1

Effect of humanized anti-PTHrP antibody on blood vasopressin level in high PTHrP-related hypercalcemia model rats

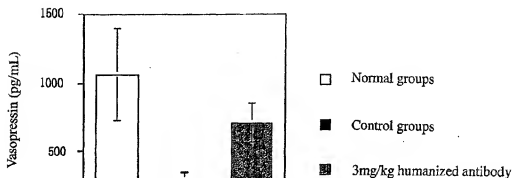
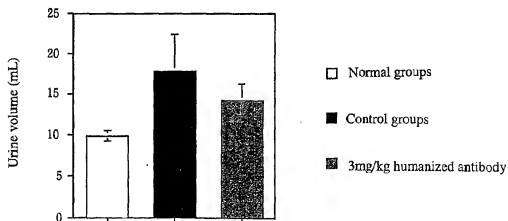


FIG. 2

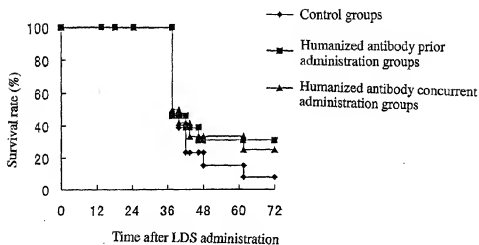
Effect of humanized anti-PTHrP antibody on urine volume in high PTHrP-related hypercalcemia model rats



EP 1 197 225 A1

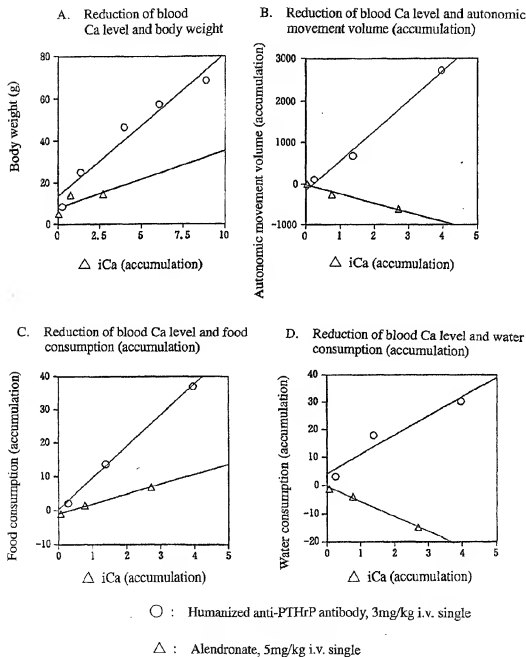
FIG. 3

Drug efficacy of humanized anti-PTHrP antibody
in septicemia models



EP 1 197 225 A1

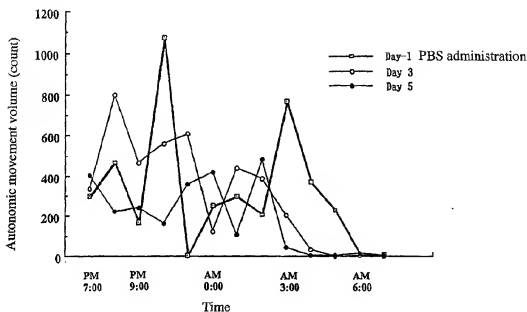
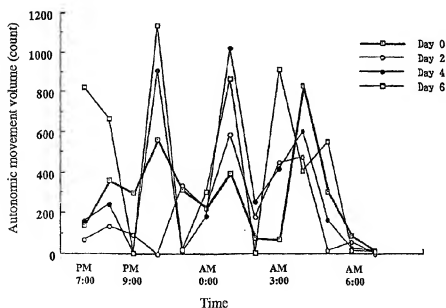
FIG. 4



EP 1 197 225 A1

FIG. 5

A. Autonomic movement volume (HHM control)

B. Autonomic movement volume
(Humanized anti-PTHrP antibody, 5mg/kg i.v.)

EP 1 197 225 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04414

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.⁷ A61K45/00, 39/395, A61P3/14, 29/00, 37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl.⁷ A61K38/00-38/32, 45/00-45/08, 39/395,
A61P3/14, 29/00, 37/00-37/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS (STN), REGISTRY (STN), MEDLINE (STN), BIOSIS (STN),
BIOTCHABS (STN), JICST (JOIS), WPI (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, 92/00753, A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 23 January, 1992 (23.01.92), Claims; page 1, lines 10 to 22	1-5, 9-11, 13, 15, 16
Y	& JP, 5-509098, A Claims; page 5, lower right column to page 6, upper left column & AU, 9182900, A & EP, 539491, A1	6-8, 12, 14
X	US, 5849695, A (The Regents of the University of California), 15 December, 1998 (15.12.98), Claims; abstract (Family: none)	1-5, 9-11, 13 6-8, 12, 14
X	WO, 98/13388, A1 (Chugai Pharmaceutical Co., Ltd.), 02 April, 1998 (02.04.98), Claims; implementation example	1-5, 9-11, 13, 15-17
Y	& JP, 11-92500, A & EP, 962467, A1 & ZA, 9708590, A & AU, 9743972, A & NO, 9901449, A & CN, 1237983, A	7, 8, 12, 14
X	JP, 11-80025, A (Chugai Pharmaceutical Co., Ltd.), 23 March, 1999 (23.03.99), Claims;	1-5, 9-11, 13, 15-17

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (or specific)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search
06 September, 2000 (06.09.00)Date of mailing of the international search report
19 September, 2000 (19.09.00)Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

EP 1 197 225 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04414

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	& WO, 98/51329, & EP, 1094313, A1 & AU, 9872369, A & NO, 9905558, A	7, 8, 12
X	WO, 95/03437, A1 (SANDOZ LTD.), 08 February, 1996 (08.02.96), Claims; page 15, line 15 to page 17, line 26	1-5, 9-11, 13
Y	& JP, 10-502091, A Claims; page 21, line 18 to page 23, line 8 & AU, 9531670, A & EP, 7739958, A1 & FI, 9700168, A & NO, 9700356, A & ZA, 9506331, A & BR, 9508433, A & KR, 97704782, A & MX, 9700446, A1	6-8, 12, 14
X	EP, 293158, A2 (MERCK & CO. INC.), 30 November, 1988 (30.11.88), Claims; page 3, line 25 to page 4, line 19	1-5, 9-11, 13
Y	& JP, 63-313800, A Claims; page 5, upper left column, line 8 to page 6, upper left column, line 17 & DK, 8802853, A	6-8, 12, 14
X	JP, 7-316195, A (NIPPON KAYAKU CO., LTD.), 05 December, 1995 (05.12.95), Claims (Family: none)	1-3, 9-11, 13
Y		6-8, 12, 14
X	WO, 96/39184, A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 12 December, 1996 (12.12.96), Claims	1-3, 9-11, 13, 15, 16
Y	& US, 5660826, A & AU, 9658844, A	7, 8, 12, 14
X	JP, 2-207099, A (Toa Nenryo Kogyo K.K.), 16 August, 1990 (16.08.90), Claims; page 1, lower right column to page 2, lower right column, line 7 (Family: none)	1, 3-5, 9-11, 13
Y		7, 8, 12, 14
X	JP, 4-228089, A (Kanegafuchi Chem. Ind. Co., Ltd.), 18 August, 1992 (18.08.92), Claims; Par. Nos. [0002], [0003], [0010] (Family: none)	1, 3-5, 9-11, 13, 15, 16
Y		6-8, 12, 14
X	JP, 7-165790, A (Tonen Corporation), 27 June, 1995 (27.06.95), Claims; Par. Nos. [0001], [0002], [0005], [0008], (Family: none)	1-5, 9-11, 13
Y		7, 8, 12, 14
X	WO, 92/17602, A1 (The General Hospital Corporation Office of Technology Affairs), 15 October, 1992 (15.10.92), Claims; page 40, line 14 to page 49, line 6	1, 3-5, 9-11, 13, 15, 16
Y	& JP, 6-506598, A. Claims; page 13, upper left column to page 15, upper left column, & EP, 579758, A1 & US, 5886148, A	6-8, 12, 14
X	WO, 96/33735, A1 (Cell Genesys, Inc.), 31 October, 1996 (31.10.96), Claims; page 16, line 23 to page 17, line 27; implementation example 7	1, 2, 15-17
Y	& JP, 11-505523, A, claims; page 24, line 10 to page 25, line 9; implementation example 7 & EP, 822830, A1, & AU, 9656322, A & KR, 99008096, A & US, 6075181, A	7, 8, 12, 14

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

EP 1 197 225 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04414

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, 878201, A1 (Chugai Seiyaku Kabushiki Kaisha),	1-3
Y	18 November, 1998 (18.11.98), Claims & JP, 8-301887, A, Claims & WO, 97/27870, A1 & AU, 9715581, A	7,8,12,14
Y	HARDMAN, J. G., et al., (ed.), "Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS-9 th ed", McGraw-Hill Companies (USA), pp.1528-1529	6
Y	Shigeto MORIMOTO et al., "PTH/PTHrP to Chusu Shinkeikei", CLINICAL CALCIUM, 5(12), pp.50-54 (1995), see the full text	7,8,12
Y	YAMAMOTO, S. et al., "Parathyroid Hormone-Related Peptide-(1-34) [PTHrP-(1-34)] Induces Vasopressin Release from the Rat Supraoptic Nucleus <i>in Vitro</i> through a Novel Receptor Distinct from a Type I or Type II PTH/PTHrP Receptor", Endocrinology, 138(5), pp.2066-2072 (1997)	14
PX	WO, 00/00219, A1 (Chugai Pharmaceutical Co., Ltd.),	1,3-5,9-11,13,
	06 January, 2000 (06.01.00),	15-17
PY	Claims; page 2, the last line to page 3, the last line, & AU, 9942899, A	7,8,12,14
PX	JP, 2000-80100, A (Japan Tobacco Inc.),	1-5,9-11,
	21 March, 2000 (21.03.00),	13-16
PY	Claims; Par. Nos. [0013], [0014], [0055], [0056]; implementation example (Family: none)	6-8,12,14
PX	WO, 99/57139, A2 (SOCIETE DE CONSBILS DE RECHERCHES ET D'APPLICATIONS SCIENTIFIQUES S.A.),	1-3,7,8,12
	11 November, 1999 (11.11.99),	
	Claims; Abstract & AU, 9936736, A	
PX	JP, 11-222440, A (Asahi Chemical Industry Co., Ltd.),	1-3
	17 August, 1999 (17.08.99),	
PY	Claims (Family: none)	14
A	Ryouji IKEDA, "Fuku Koujousen Hormone Kanren Peptide no Bunshi Seibutsugaku", Nihon Rinshou, 53(4), 1995, pp.37-45, Hajimeni, IV. Akusei Shuyou to PTHrP	1-17
A	ROSEN, H.N. et al., "The Effect of PTH Antagonist BIM-44002 on Serum Calcium and PTH Levels in Hypercalcemic Hyperparathyroid Patients", Calcif. Tissue Int., 61, pp.455-459 (1997)	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

EP 1 197 225 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04414

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(See extra sheet)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

EP 1 197 225 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/04414

Continuation of Box No.II of continuation of first sheet (1)

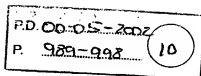
Invention as set forth in claim 1 pertains to remedies for diseases caused by PTH or PTHrP which contain, as the active ingredient, an agonist or an antagonist binding to PTH receptor or PTHrP receptor or a substance binding to a ligand of such a receptor to thereby promote or inhibit the binding of the ligand to the receptor. As described in the documents (JP, 6-506598 A, JP, 4-228089, A, JP, 5-509098, A, etc.) cited in the description by the applicant, remedies for these diseases with the use of such ingredients had been widely known. Therefore, it is understood that the "special technical feature" in the present invention resides in particular diseases caused by PTH or PTHrP.

It is recognized that claims 1 to 17 in the present application pertain to groups of inventions 1) to 5) as shown below. However, it is not considered that there is a "special technical feature" among these groups of inventions. Such being the case, these groups of inventions are considered as not complying with the requirement of unity of invention:

- 1) claims 1, 2 and 6 and the parts in claims 14 to 17 depending on claims 1, 2 and 6;
- 2) claim 3 and the parts in claims 14 to 17 depending on claim 3;
- 3) claims 4 and 5 and the parts in claims 14 to 17 depending on claims 4 and 5;
- 4) claims 7, 8 and 12 and the parts in claims 14 to 17 depending on claims 7, 8 and 12; and
- 5) claims 9 to 11 and 13 and the parts in claims 14 to 17 depending on claims 9 to 11 and 13.



XP-002237380



PEPTIDES

Peptides 23 (2002) 989-998

Specificity and stability of a new PTH1 receptor antagonist, mouse TIP(7-39)

Sam R.J. Hoare, Ted B. Usdin*

Unit on Cell Biology, National Institute of Mental Health, Room 3D06, Bld. 36, 36 Convent Drive, Bethesda, MD 20892-4062, USA

Received 11 October 2001; accepted 27 November 2001

Abstract

Parathyroid hormone 1 (PTH1) receptor antagonists might be of benefit in hypercalcemia of malignancy (HHM) and hyperparathyroidism. We previously identified bovine tuberoinfundibular peptide (7-39) (bTIP(7-39)) as a high-affinity PTH1 receptor antagonist. Mouse TIP(7-39) is an antagonist (rPTH1 K_D = 44 nM, rPTH2 = 940 nM) that is more potent than other known PTH1 receptor antagonists: bTIP(7-39) (210 nM), PTH-related protein (PTHrP)(7-34) (640 nM), and bPTH(7-34) (>3000 nM). Plasma proteases slowly ($t_{1/2}$ = 81 min) inactivated [125 I]mTIP(7-39). Intravenous plasma [125 I]mTIP(7-39) was bi-phasically cleared (radioactivity $t_{1/2}$ = 2.9 min (70%) and 120 min (30%); binding activity $t_{1/2}$ = 3.6 min (92%), and $t_{1/2}$ = 21 min (8%). Loss of unlabeled mTIP(7-39) (250 μ g/kg i.v.) receptor binding was similar. mTIP(7-39)'s high-affinity should facilitate animal evaluation of effects of PTH1 receptor antagonism. Published by Elsevier Science Inc.

Keywords: Hypercalcemia; Parathyroid hormone; Tuberoinfundibular peptide; Hyperparathyroidism; Plasma pharmacokinetics

1. Introduction

Two major disorders of calcium metabolism involve elevated levels of ligands that stimulate the parathyroid hormone 1 (PTH1) receptor. Hypercalcemia of malignancy (HHM) results from excessive production of PTH-related protein (PTHrP) by certain tumors [11], and primary hyperparathyroidism (HPT) involves over-production of PTH by the parathyroid gland [26,38]. In both of these conditions hypercalcemia results through activation of the PTH1 receptor [21], which is highly expressed in bone and kidney [21,29].

HHM and HPT can be treated by inhibiting the physiological effects of PTH1 receptor activation in bone. The highly successful bisphosphonate drugs act by preventing bone resorption [31]. Estrogens have also been used for this purpose [39]. An alternative anti-resorptive agent in development is osteoprotegerin, which inhibits osteoblast-stimulated osteoclast production and activity [3]. However, the effect of bisphosphonates are not evident until several days after initiation of treatment [37] and their use is limited in certain clinical situations [39]. Anti-resorptive therapy also does not target renal effects of PTH and PTHrP. Block-

ing PTH1 receptor activation could circumvent these limitations. Current therapies in development lower the activity or circulating level of PTH1 receptor ligands. Anti-PTHrP antibodies lower serum calcium to the physiological range within 24 h in nude mice bearing PTHrP-producing tumors [33]. Compounds that activate calcium-sensing receptors in the parathyroid reduce serum PTH and Ca^{2+} levels within hours in rats [9] and in patients with HPT [36]. Hypercalcemia in rodents is also effectively inhibited by anti-sense or drug-induced blockade of PTHrP expression or processing in transplanted tumor cells [12,24,30]. Blocking PTH1 receptor activation could also inhibit actions of PTHrP in HHM beyond hypercalcemia. Cachexia associated with malignancy involves pro-inflammatory cytokine production stimulated by PTHrP, an effect that is ameliorated with an anti-PTHrP antibody [27]. PTHrP activation of the PTH1 receptor is involved in proliferation of tumorigenic breast epithelial cells *in vitro* [4]. In addition, anti-sense or drug-induced blockade of PTHrP reduces tumor volume in animals injected with certain tumor cells [12,24,30], and reduces angiogenesis in implants of malignant GH3 cells [1]. However, the extent to which the PTH1 receptor is involved in these latter effects is not known; fragments of PTHrP act directly in the nucleus [23] and on other, as yet-identified receptors [38].

PTH1 receptor antagonism could provide an alternative means to block PTH1 receptor activation by elevated

* Corresponding author. Tel.: +1-301-402-6976; fax: +1-301-435-5456. E-mail address: usdin@codon.nih.gov (T.B. Usdin).

PTII and PTHrP. Peptide antagonists derived from PTII and PTHrP block hypercalcemia induced by infused PTH or PTHrP in thyroparathyroidectomized animals [7,8,17,18]. However, these ligands were ineffective in an animal model of HHM [22] or in human subjects with HPT [32]. However, these latter studies have a number of limitations. First, the most-studied ligand, BIM-44002 ($[\text{Nle}^{8,18}, \text{D-Trp}^{12}, \text{Tyr}^{34}] \text{bPTH(7-34)}-\text{NH}_2$, where b is bovine), has a very short plasma half-life in rats; no intact $[\text{I}^{125}]\text{BIM-44002}$ could be detected in plasma 20 min after administration, assessed by HPLC [34]. Second, only low doses of BIM-44002 were tested in the human study (predicted antagonist:PTH ratio of 2:1 [32]). In a mouse model of HHM the dose of $[\text{D-Trp}^{12}, \text{Tyr}^{34}] \text{bPTH(7-34)}$ and $[\text{Tyr}^{34}] \text{bPTH(7-34)}$ was a 500-fold excess of a dose of PTHrP(1–34) known to produce hypercalcemia [22]. However, the K_i of these ligands for antagonism of the PTH1 receptor in ROS 17/2.8 cells (210 and 2700 nM, respectively [5]) is 3–4 orders of magnitude greater than the EC_{50} of PTHrP(1–34) for stimulating cAMP accumulation in these cells. Third, $[\text{D-Trp}^{12}, \text{Tyr}^{34}] \text{bPTH(7-34)}$ and PTHrP(7–34) dissociate very rapidly from the human PTH1 receptor ($t_{1/2}$ values of about 10 s, calculated from dissociation rate constants [15]), and likely dissociate more rapidly from the rat PTH1 receptor given their 30–100-fold lower binding affinity compared with the human receptor [35]. Rapid antagonist degradation and dissociation rates, coupled with high concentrations of agonist and slow agonist dissociation from the receptor [13], might result in considerably lower receptor occupancy by the antagonist than predicted from a simple equilibrium model. (These kinetic considerations might explain, in part, the requirement of pre-administration of antagonist peptide in order to detect antagonism of injected PTH in thyroparathyroidectomized rodents [8].) A more potent antagonist might demonstrate an anti-hypercalcemic effect in animal models of hypercalcemia.

High-affinity PTH1 receptor antagonists have been derived from N-terminal truncation of the PTH12 receptor's endogenous ligand, tuberointerfundular peptide of 39 residues (TIP39) [15,19,41]. bTIP(7–39) binds with high affinity to the human PTH1 receptor (6 nM), is an order of magnitude more potent than $[\text{D-Trp}^{12}, \text{Tyr}^{34}] \text{bPTH(7-34)}$ and PTHrP(7–34), and dissociates much more slowly from the receptor ($t_{1/2}$ of 14 min) [15]. The *in vivo* activity of TIP(7–39) will likely be tested in rodents, so the first aim of this study was to evaluate the activity of TIP(7–39) on the rat PTH1 and PTH12 receptors. Included in this evaluation was mouse (m) TIP(7–39), differing from the bovine sequence by four amino acids in the C-terminal portion of the peptide [10]. The second aim was to investigate the plasma lifetime of mTIP(7–39), in order to evaluate the extent to which it could be used to test the utility of PTH1 receptor antagonism in rodent models of hypercalcemia.

2. Methods

2.1. Reagents and peptides

bTIP(7–39), mTIP(7–39) amide, and bTIP were from Bioimolecules Midwest (Waterloo, IL), and $[\text{Tyr}^{34}] \text{bPTH(7-34)}$ amide, PTHrP(7–34) amide, and PTHrP(1–34) were from Bachem (Torrance, CA). Peptides were dissolved in 10 mM acetic acid at a concentration of 1 mM. $[\text{I}^{125}]\text{bTIP(7-39)}$ was prepared by the lactose peroxidase method followed by HPLC purification [14]. For *in vivo* injection, aliquots of $[\text{I}^{125}]\text{mTIP(7-39)}$ ($2\text{--}9 \times 10^6$ cpm) were dried in a vacuum centrifuge, removing trifluoroacetic acid (TFA) and acetonitrile, then re-suspended in 50 μl sterile 0.1% BSA in saline. Cell culture supplies were from Life Technologies (Frederick, MD). DMEM and Ham's F-11 medium were from Mediatech (Herndon, VA).

2.2. Cell culture and transient expression in COS-7 cells

COS-7 cells were grown as previously described [6]. COS-7 cells were transfected as previously described [6], using 10 cm tissue culture dishes and 10 μg of plasmid DNA. The following day cells were transfected following trypsinization to 96-well plates at a density of 50,000 cells per well. HEK293 cells stably expressing the cloned human PTH1 receptor or human PTH2 receptor were grown as previously described [40]. ROS 17/2.8 cells were grown in tissue-culture flasks in Ham's F-12 medium, 5% fetal bovine serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. For cAMP accumulation assays ROS 17/2.8 cells were transferred 2 days prior to assay into 96-well plates at 20,000 cells per well.

2.3. Measurement of cellular levels of cAMP

Transfected COS-7 cells were treated for 30 min at 37°C with 50 μl per well cAMP assay buffer (DMEM containing 25 mM HEPES supplemented with 0.1% BSA, 30 μM Ro 20-1724 (RBI, Natick, MA), 100 μM (4-(2-aminoethyl)-benzencysulfonyl fluoride (AEBSF) and 1 $\mu\text{g}/\text{ml}$ bacitracin). This buffer was then removed and replaced with 40 μl fresh buffer. A 10 μl volume of buffer was then added containing either PTHrP(1–34) alone or PTHrP(1–34) and antagonist ligand. Cells were then incubated for 30 min at 37°C prior to assay termination by addition of 50 μl 0.1N HCl, 0.1 mM CaCl_2 . The same protocol was used for measurement of cAMP in ROS 17/2.8 cells, except that the volumes were doubled. cAMP was quantified by ELISA [6].

2.4. Measurement of equilibrium radioligand binding to cell membrane preparations

Membranes were prepared from HEK293 cells stably expressing the human PTH1 receptor or PTH2 receptor

[16]. The following were added to polypropylene V-bottom 96-well plates (Nunc, Naperville, IL): 25 μ l assay buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 3 mM $MgSO_4$, pH 7.5, supplemented with 0.3% non-fat dried milk powder, 100 μ M AEBSP and 1 μ g/ml bacitracin), 25 μ l [125 I]mTIP(7–39) in buffer, 25 μ l unlabeled ligand in buffer, and 50 μ l membrane suspension. Total [125 I]mTIP(7–39) binding was measured by replacing unlabeled ligand with buffer alone and non-specific binding measured using 1 μ M mTIP(7–39). The mixture was incubated at 21 °C for 1 h, prior to separation of bound and free radioligand by rapid filtration over MAHV N45 MultiScreen filtration plates (Millipore, Bedford, MA) [16]. Filter-bound [125 I] was measured in a Wallac 1470 Wizard gamma counter for 1 min. Equilibrium binding of mTIP(7–39) was also measured in the presence of rat plasma, using the protocol above, replacing the 25 μ l assay buffer with plasma. PTH1 receptor binding activity in plasma from rats injected with unlabeled mTIP(7–39) was measured using the protocol above, replacing unlabeled ligand with the plasma. The plasma binding activity in rats injected with [125 I]mTIP(7–39) was measured by replacing the 25 μ l radioligand in buffer with plasma. In this assay total binding and non-specific binding were measured, as described, and filters were counted for 20 min due to the low number of counts recovered at later time points. In all assays that included plasma, AEBSP and bacitracin concentration was raised to 500 μ M and 5 μ g/ml, respectively.

The effect of rat plasma *in vitro* on [125 I]mTIP(7–39) binding to the PTH1 receptor was measured in a time course experiment. Aliquots of 25 μ l EDTA-treated plasma (see below) or assay buffer were added to 25 μ l aliquots of [125 I]mTIP(7–39) in buffer in 0.65 ml siliconized microfuge tubes. The mixture was pre-incubated for varying times at 37 °C and then total and non-specific binding was measured in duplicate using 10 μ l of the mixture as the tracer in an equilibrium PTH1 receptor binding assay, as described. Four conditions were used in the pre-incubation step: (1) plasma without protease inhibitors; (2) plasma with protease inhibitors (500 μ M AEBSP, 5 μ g/ml bacitracin); (3) assay buffer without protease inhibitors; (4) assay buffer with protease inhibitors. Initiation of the pre-incubation phase was staggered so that the pre-incubation mixture could be added simultaneously to the binding assay. Prior to their mixture, [125 I]mTIP(7–39) in buffer was maintained at room temperature and rat plasma was maintained at 4 °C.

2.5. Intravenous injection of peptides into rats

The animal protocol was approved by the NIMH Animal Care and Use Committee. Male 300–400 g rats were anaesthetized with 0.5 mg/kg sodium pentobarbital. Canulae were fitted into the jugular vein and the dorsal tail artery. The arterial line was treated with heparin (100 U/ml). Before injection, 0.5 ml blood was drawn from the artery.

Peptide was then injected intravenously, either 100 μ g unlabeled mTIP(7–39) (dissolved in 25 μ l water then diluted in 1 ml saline) or 15–80 million cpm of [125 I]mTIP(7–39) (prepared as described in Section 2.1, diluted in 0.8 ml saline). After discarding the first six drops, 0.5 ml blood was collected at various times by allowing it to drip from the arterial line. Blood was collected into 1.7 ml siliconized microfuge tubes containing 15 μ l 0.5 M di-sodium EDTA pH 8.0 (final concentration in blood of 15 mM), then centrifuged at 1000 \times g for 10 min at 4 °C. For binding experiments, plasma was removed and frozen at –20 °C. For analysis of [125 I]mTIP(7–39) in plasma, a plasma aliquot was removed for counting of radioactivity and an aliquot was immediately treated as described in Section 2.6.

2.6. Reversed-phase HPLC of plasma from rats injected with [125 I]mTIP(7–39)

One hundred microliter plasma was treated with 100 μ l 8% TFA or with 0.9 ml Bennett's cocktail [2] (1 M HCl, 5% formic acid, 1% NaCl, and 1% TFA), then maintained at 4 °C for 10 min before freezing at –20 °C. After thawing the precipitate was removed by centrifugation at 23,000 \times g for 10 min at 4 °C. The supernatant from the Bennett's solution precipitation was loaded directly onto a Vydac C4 column pre-equilibrated with 80% buffer A (0.1% TFA in H_2O)/20% buffer B (60% acetonitrile, 0.1% TFA in H_2O) at 1 ml/min. The supernatant from TFA precipitation was diluted with 0.6 ml buffer A and 0.2 ml buffer B before loading onto the column. The column run-through was immediately collected in fractions of 1 min. After 12 min the column was eluted with three sequential linear gradients at 1 ml/min: 20–50% B over 1 min; 50–67.5% B over 35 min; 67.5–100% B over 1 min. One minute fractions were collected and radioactivity in the entire fraction counted.

2.7. Data analysis

Concentration-dependence data were analyzed using the following four parameter-logistic equation using Prism 2.01 (GraphPad Software Inc., San Diego, CA):

$$y = \min + \frac{\max - \min}{1 + 10^{(K-X)}}$$

where X is the logarithm of the ligand concentration, π the Hill slope, and K is $\log EC_{50}$ for cAMP accumulation or $\log IC_{50}$ for inhibition of radioligand binding.

Antagonism of agonist-stimulated cAMP accumulation was quantified using the pK_B , the negative logarithm of the concentration of antagonist required to produce a two-fold increase of agonist EC_{50} :

$$pK_B = \log \frac{EC_{50}(\text{+antagonist})}{EC_{50}(\text{control})} - \log [\text{antagonist}]$$

The time course of labeled and unlabeled mTIP(7–39) activities in plasma were fitted to the following

mono-exponential and bi-exponential decay functions and the best fit determined using a partial *F*-test.

$$P_t = \text{plateau} + P e^{-k_1 t}$$

$$P_t = \text{plateau} + P_{\text{(FAST)}} e^{-k_1 \text{(FAST)} t} + P_{\text{(SLOW)}} e^{-k_1 \text{(SLOW)} t}$$

where P_t is the activity remaining after time t , P is the initial activity, and k_1 is the rate constant. In the bi-exponential decay function the subscripts (FAST) and (SLOW) refer respectively to the faster and slower independent exponential components. Plateau is the activity level remaining as t approaches infinity. In some of the analyses this value was fixed at zero.

Statistical comparison of two means was performed using a paired two-tailed Student's *t*-test.

3. Results

3.1. Antagonism of rat PTH receptors by bTIP(7–39) and mTIP(7–39)

We previously characterized the *in vitro* pharmacology of bTIP39 as an antagonist of the human PTH1 receptor [15]. In addition bTIP(7–39) was found to antagonize the rat PTH1 receptor expressed in COS-7 cells. Here we investigated antagonism of rat PTH1 receptors more extensively, since the *in vivo* activity of the antagonist will likely be tested in rodents. Mouse TIP(7–39) was also tested in an attempt to identify a higher-potency antagonist.

For the rat PTH1 receptor expressed in COS-7 cells, bTIP(7–39) and mTIP(7–39) produced a rightward shift of the PTHrP(1–34) concentration–response curve for stimulation of cAMP accumulation (Fig. 1A). The affinity of the antagonist was evaluated using the K_B , the concentration of antagonist required to produce a two-fold increase of PTHrP(1–34)'s EC_{50} . mTIP(7–39) was 4.8-fold more potent as an antagonist ($K_B = 44$ nM) than bTIP(7–39) on the rat PTH1 receptor (Table 1). The potency of bTIP(7–39) for antagonism of the PTHrP(1–34) response (210 nM) is in good agreement with the previously determined potency for antagonism of the PTH(1–34) response (310 nM) [15]. PTHrP(7–34) and PTH(7–34) were much less potent than the TIP(7–39) peptides as antagonists of the rat PTH1 receptor (Table 1). The potencies of PTHrP(7–34) and PTH(7–34) were similar to those reported previously for the rat PTH1 receptor [25]. The antagonist effect of mTIP(7–39) was then tested on the PTH1 receptor endogenously expressed in the rat osteosarcoma cell line ROS 17/2.8 (Fig. 1B). The K_B value of mTIP(7–39) (65 nM) was in good agreement with the value for the rat PTH1 receptor expressed in COS-7 cells (44 nM) (Table 1). mTIP(7–39) was also a potent antagonist of the human PTH1 receptor expressed in COS-7 cells (100 nM), although bTIP(7–39) was similarly potent to mTIP(7–39) at this receptor (150 nM, Table 1), unlike at

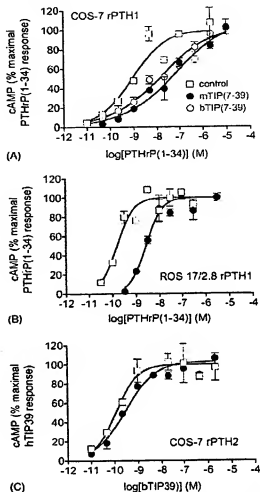


Fig. 1. Antagonist activity of mTIP(7–39) and bTIP(7–39) on rat PTH1 and PTH2 receptors. Antagonist activity was evaluated by measuring the effect of a single concentration of peptide on the agonist concentration response for stimulation of cAMP accumulation. Antagonist ligands mTIP(7–39) and bTIP(7–39) were tested at a concentration of 1 μ M (A) on the rat PTH1 receptor over-expressed in COS-7 cells; (B) on the rat PTH1 receptor endogenously expressed in ROS 17/2.8 cells; and (C) on the rat PTH2 receptor expressed in COS-7 cells. The agonists used were PTHrP(1–34) for the PTH1 receptor and bTIP39 for the PTH2 receptor. Data are normalized as the percentage of the maximal agonist-specific response in the absence of antagonist. Data points are the mean \pm range of duplicate measurements. The experiments were performed three times, except for the mTIP(7–39) effect on the PTH2 receptor, which was performed twice.

the rat receptor. The selectivity of mTIP(7–39) was tested by comparing its antagonist potency for the rat PTH1 and PTH2 receptors expressed in COS-7 cells. mTIP(7–39) produced a measurable rightward shift of the bTIP39 concentration response on the rat PTH2; however, the shift for the rat PTH1 receptor was much greater than the shift for the PTH2 receptor (compare data for 1 M concentrations in Figs. 1A and C). However, the potency of mTIP(7–39) (940 nM) was 21-fold lower than its potency to antagonizing the rat PTH1

Table 1
Activity of antagonist ligands on rat and human PTH1 and PTH2 receptors^a

Ligand	Rat PTH1 receptor COS-7 cells		Rat PTH1 receptor ROS 17/2.8 cells		Human PTH1 receptor COS-7 cells		Rat PTH2 receptor COS-7 cells	
	pK _d (K _d , nM)	$\frac{E_{max}-ligand^b}{E_{max}-control}$ (%)	pK _d (K _d , nM)	$\frac{E_{max}-ligand^b}{E_{max}-control}$ (%)	pK _d (K _d , nM)	$\frac{E_{max}-ligand^b}{E_{max}-control}$ (%)	pK _d (K _d , nM)	$\frac{E_{max}-ligand^b}{E_{max}-control}$ (%)
mPTH(7–39)	7.36 ± 0.21 (44) ^c	95 ± 12	7.19 ± 0.19 (65)	115 ± 5	6.68 ± 0.22 (100)	88 ± 6	6.03 ± 0.19 (940)	110 ± 7
hPTH(7–39)	6.68 ± 0.19 (210) ^b	99 ± 4			6.82 ± 0.22 (150)	103 ± 11		
mPTH(7–34)	6.20 ± 0.02 (640)	103 ± 9						
hPTH(7–34)	>5.5 (>3200) ^c							

^a Antagonist potency was evaluated by measuring the shift of agonist EC₅₀ for cAMP accumulation produced by a fixed concentration of antagonist, converted to the pK_d value as described in Section 2.7. The agonists used were PTHrP(1–34) for the PTH1 receptors and DT130 for the rat PTH2 receptor. Antagonists were tested at a concentration of 1 μM, except hPTH(7–34) which was tested at 3.2 μM and mPTH(7–39) at the rat PTH2 receptor tested at 1 μM in two experiments and at 3.2 μM in one experiment. Values are the mean ± S.E.M. of data from three experiments or mean ± range from two experiments.

^b The pK_d values are significantly different ($P = 0.005$).

^c hPTH(7–34) did not produce a detectable shift of the PTHrP(1–34) concentration-response curve so it is assumed that the K_d is greater than 3.2 μM.

receptor (Table 1). In all of the experiments above, the antagonists behaved competitively since the maximal agonist response was not affected (Table 1). These findings indicate that mTIP(7–39) is a highly potent and relatively selective competitive antagonist of the rat PTH1 receptor.

3.2. *In vitro* effect of rat plasma on the bioactivity of [125 I]mTIP(7–39)

The next aim was to evaluate the plasma lifetime of TIP(7–39). mTIP(7–39) was used since it was more potent than bTIP(7–39) on the rat PTH1 receptor (Table 1). We first evaluated the stability of [125 I]mTIP(7–39) in rat plasma *in vitro*, by measuring its binding to the human PTH1 receptor in HEK293 cell membranes after pre-incubation with rat plasma at 37°C. ([125 I]mTIP(7–39) binds the human PTH1 receptor in HEK293 cell membranes with a signal-to-background ratio of 5:1, with an affinity of 3.0 nM, and with a dissociation rate $t_{1/2}$ of 6.3 min (data not shown).) It should be noted that in this experiment plasma was treated with EDTA (15 mM) to prevent clotting, which inhibits Ca^{2+} - and Mg^{2+} -dependent protease activity.

Pre-incubation with rat plasma reduced specific binding of [125 I]mTIP(7–39) to the PTH1 receptor in a time-dependent fashion (Fig. 2). The effect was specific for plasma since pre-incubation with buffer did not reduce specific radioligand binding (Fig. 2). Protease inhibitors blocked the reduction of binding produced by plasma (Fig. 2), strongly suggesting that plasma proteases were responsible for the

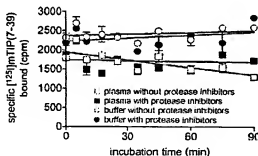


Fig. 2. Time course of [125 I]mTIP(7–39) plasma bioactivity *in vitro*. The experiment consisted of two incubations. In the first, [125 I]mTIP(7–39) was incubated with rat plasma or buffer, in the absence or presence of protease inhibitors, for the indicated times at 37°C. The mixture was then used as the tracer in a human PTH1 receptor radioligand binding assay to measure the binding activity remaining after the first incubation. Radioligand and membranes bearing the human PTH1 receptor were incubated at 21°C for 1 h in the presence of protease inhibitors. Specific binding was calculated by subtracting non-specific binding, that measured in the presence of 1 μ M unlabeled mTIP(7–39). Non-specific binding was similar when measured using radioligand incubated for 0 or 90 min in the first incubation. The mean \pm range of these two values was 361 ± 18 and 375 ± 14 cpm for plasma without and with protease inhibitors, respectively, and 318 ± 11 and 397 ± 32 cpm for buffer without and with protease inhibitors, respectively. Data points are the mean \pm S.E.M. of duplicate measurements. The experiment was performed twice with similar results.

reduction of bioactivity. The rate of this reduction was determined by fitting the data to a mono-exponential decay function, extrapolating the asymptote for specific binding to zero. The rate constant was $0.0085 \pm 0.0047 \text{ min}^{-1}$ (mean \pm range, $n = 2$, $t_{1/2} = 81 \text{ min}$). This indicates the bioactivity of [125 I]mTIP(7–39) is not greatly affected by rat plasma *in vitro*, under conditions in which Ca^{2+} - and Mg^{2+} -dependent proteases are suppressed.

3.3. *In vivo* plasma stability of [125 I]mTIP(7–39)

In these experiments [125 I]mTIP(7–39) was injected intravenously into anaesthetized rats. Plasma collected at various times was used in three ways: (1) clearance of radioactivity was determined by counting an aliquot of plasma; (2) bioactivity was evaluated using plasma as the radioligand tracer in a human PTH1 receptor binding assay; (3) the nature of radioactivity was analyzed by reversed-phase HPLC, following precipitation and removal of high-molecular weight components.

Clearance of [125 I]mTIP(7–39) radioactivity was bi-phasic (Fig. 3A, Table 2). The rapid component of the time course likely approximates the rate of radioligand absorption ($t_{1/2}$ of 2.9 min, 70% of total activity) and the slower rate may represent elimination ($t_{1/2}$ of 120 min, 30% of total activity). A radioactive component in plasma bound specifically to the PTH1 receptor in HEK293 cell membranes (Fig. 3B). No binding was observed to membranes prepared from non-transfected HEK293 cells (data not shown). Disappearance of this bioactivity over time was bi-phasic. Most PTH1 receptor binding activity was removed rapidly (92%, $t_{1/2}$ of 3.6 min) but a significant fraction was removed considerably more slowly (8%, $t_{1/2}$ of 21 min). Under the conditions of the binding assay, the level of PTH1-receptor bound [125 I]mTIP(7–39) is essentially linearly proportional to the level in the plasma, because the total amount of radioactivity in the assay (6–25 pM) was much lower than the K_D of the ligand (1.7 nM, measured in plasma, see below). As a result, the reduction of PTH1 receptor binding activity closely approximates the reduction of the plasma concentration of bioactive radioligand.

HPLC analysis of plasma radioactivity revealed three peaks, one in the column run-through and two during the organic solvent gradient (Fig. 3C). Upon chromatography of the [125 I]mTIP(7–39) stock solution used for injection (in 0.1% bovine serum albumin (BSA)/saline), a small amount of radioactivity was detected in the column run-through and two peaks eluted from the column in the same fractions as those in plasma. Radioactivity in the run-through likely represents low-molecular weight degradation products. The first peak eluted during the gradient likely corresponds to [125 I]mTIP(7–39) since it is eluted at the same position as the radioligand purified following iodination. The nature of the second, smaller peak is not known. It may be the radioligand in complex with BSA or plasma proteins.

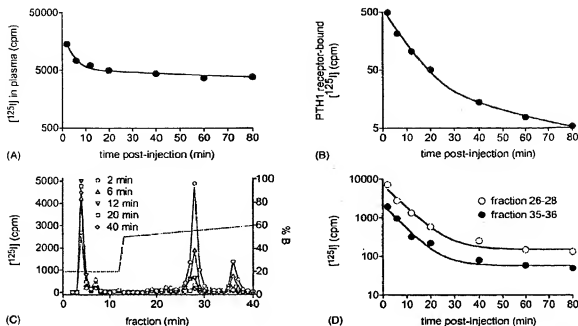


Fig. 3. Time course of $[^{125}\text{I}]$ mTIP(7-39) plasma activity in vivo. $[^{125}\text{I}]$ mTIP(7-39) was injected into rats (7.7×10^7 cpm in this experiment), blood was collected at the indicated time points and the blood cells removed as described in Section 2.5. (A) Time course of $[^{125}\text{I}]$ in rat plasma. Ten microliter plasma was counted at each time point. The curve is a bi-exponential decay function, which provided a significantly better fit to the data ($P = 0.0005$) than a mono-exponential function. The plateau value was fixed at zero in this analysis. (B) Time course of $[^{125}\text{I}]$ PTH1 receptor binding activity. Twenty-five microliter plasma from rats injected with $[^{125}\text{I}]$ mTIP(7-39) was incubated with the human PTH1 receptor as described in Section 2.4. Non-specific binding was measured for plasma from each time point by including $1 \mu\text{M}$ unlabeled mTIP(7-39) and total binding was measured in the absence of unlabeled ligand. PTH1-receptor bound $[^{125}\text{I}]$ was calculated by subtracting non-specific binding from total binding. Due to the low number of counts bound at later time points, radioactivity was counted for 20 min. At 80 min total binding was 493 ± 6 counts in 20 min and non-specific binding was 385 ± 15 counts in 20 min. Data points are the mean \pm range of duplicate measurements (error bars are hidden by the symbols). The curve is a bi-exponential decay function, which provided a significantly better fit to the data ($P = 0.0095$) than a mono-exponential function. The plateau value was fixed at zero in this analysis. (C) HPLC analysis of supernatants from plasma treated with Bennett's cocktail. This solution precipitates high-molecular weight components in plasma. The supernatant from treated plasma collected at the indicated time points was analyzed by reversed-phase HPLC, as described in Section 2.6. Both the eluted peaks in the chromatogram of the treated plasma were also identified in the chromatogram of $[^{125}\text{I}]$ mTIP(7-39) stock (data not shown). Data points are single measurements of the entire fraction. (D) Time course of $[^{125}\text{I}]$ in eluted peaks of activity. The curves are mono-exponential decay functions, the bi-exponential fit providing no improvement ($P = 0.27$ for fraction 26-28 peak and $P = 0.52$ for fraction 35-36 peak). The plateau was not fixed in the analysis. The entire experiment was performed three times, except that measurement of receptor-bound $[^{125}\text{I}]$, (B) was performed in two of the three experiments. Note the logarithmic scale of the y-axis in A, B and D.

Table 2

Plasma pharmacokinetics and bioactivity of $[^{125}\text{I}]$ mTIP(7-39) and unlabeled mTIP(7-39)^a

Measured activity	$k_{1(\text{FAST})}$ (min^{-1})	$(t_{1/2}, \text{min})$	$P_{(\text{FAST})}$ (%)	$k_{1(\text{SLOW})}$ (min^{-1})	$(t_{1/2}, \text{min})$	$P_{(\text{SLOW})}$ (%)
Plasma radioactivity	0.24 ± 0.02 (2.9)		70 ± 3	0.0056 ± 0.0012 (120)		30 ± 3
IPLC peak 1 radioactivity	0.20 ± 0.06 (3.4)		100			
HPLC peak 2 radioactivity	0.18 ± 0.05 (3.9)		100			
$[^{125}\text{I}]$ mTIP(7-39) binding activity	0.19 ± 0.04 (3.6)		92 ± 3	0.033 ± 0.011 (21)		8 ± 3
mTIP(7-39) binding activity	0.28 ± 0.10 (2.5)		96 ± 1	0.035 ± 0.007 (20)		4 ± 1

^a The antagonist was injected intravenously into rats. The dose of $[^{125}\text{I}]$ mTIP(7-39) was between 1.4×10^7 and 8.1×10^7 cpm (31 – 180 ng/kg) and the dose of unlabeled antagonist was $250 \mu\text{g/kg}$. Radioactivity and bioactivity were measured as described in Figs. 3 and 4 and in Section 2. The time course data were described by a bi-exponential decay function, except for the clearance of HPLC peak activity, which was described by a mono-exponential function. The bi-exponential function is defined by two rate constants ($k_{1(\text{FAST})}$ and $k_{1(\text{SLOW})}$) and by the proportions of the total activity that decays at these rates ($P_{(\text{FAST})}$ and $P_{(\text{SLOW})}$, respectively). Values are the mean \pm S.E.M. from three experiments, except for the binding activity of $[^{125}\text{I}]$ mTIP(7-39) where the values are the mean \pm range from two experiments.

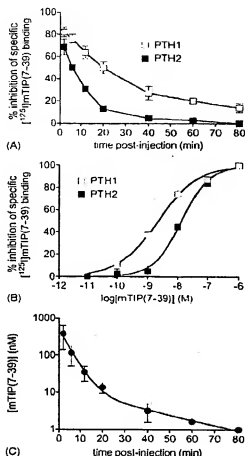


Fig. 4. Time course of unlabeled mTIP(7–39) plasma bioactivity in vivo. (A) Inhibition of $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to PTH1 and PTH2 receptors by plasma from rats injected with mTIP(7–39). mTIP(7–39) was injected into rats intravenously (250 $\mu\text{g}/\text{kg}$), blood was collected at the indicated time points and the blood cells removed as described in Section 2.5. The bioactivity was evaluated by measuring the ability of 25 μl of the plasma samples to inhibit $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to the human PTH1 or PTH2 receptor in HEK293 cell membranes (in a total volume of 125 μl). The PTH2 receptor was included to assess the pharmacological specificity of the plasma activity. Data were normalized as the percentage inhibition of specific binding measured in the presence of plasma drawn from the rat prior to injection of mTIP(7–39). Data points are the mean \pm S.E.M. of data from three independent experiments. In each experiment triplicate measurements of binding inhibition were made for a single plasma sample at each time point. (B) Standard curve of inhibition of $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to human PTH1 and PTH2 receptors by mTIP(7–39) in the presence of plasma. mTIP(7–39) was diluted in rat plasma (from an animal not injected with the peptide) and assayed as described above. Data points are the mean \pm range from two independent experiments. The $-\log\text{IC}_{50}$ value for mTIP(7–39) was 8.77 ± 0.07 for the PTH1 receptor and 7.84 ± 0.05 for the PTH2 receptor. (C) Time course of the calculated plasma concentration of mTIP(7–39). The standard curve for the PTH1 receptor. (B) was used to calculate the effective concentration of mTIP(7–39) in the plasma from rats injected with the peptide (A). The curve is a bi-exponential decay function, which provided a significantly better fit to the data ($P < 0.0001$) than a mono-exponential function in each of three assays. The plateau value was fixed at zero in these analyses. Average values of the fitted parameters are given in Table 2. Data points are the mean \pm S.E.M. of data from three independent experiments. Note the logarithmic scale of the y-axis.

The time course of the disappearance of these peaks is shown in Fig. 3C and D. Disappearance of the peak corresponding to free intact $[^{125}\text{I}]\text{mTIP}(7-39)$ (fractions 26–28) was described by a mono-exponential decay function ($t_{1/2}$ of 3.4 min, Fig. 3D, Table 2). A similar time course described the disappearance of the second peak eluted from the column ($t_{1/2}$ of 3.9 min Fig. 3D, Table 2). The activity in the column run-through increased then reached a plateau at 12 min (Fig. 3C).

3.4. In vivo plasma stability of unlabeled mTIP(7–39)

Bioactivity of the unlabeled antagonist was evaluated in plasma samples taken from anaesthetized rats at various times following intravenous administration of a single dose of mTIP(7–39) (250 $\mu\text{g}/\text{kg}$). Bioactivity was measured as the ability of the plasma sample to inhibit $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to the human PTH1 receptor in HEK293 cell membranes.

PTH1 receptor binding activity decreased with time after injection of the peptide (Fig. 4A). Significant binding activity could still be detected at 80 min (Fig. 4A). The pharmacological specificity of the binding activity was evaluated by measuring its ability to block $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to the human PTH2 receptor. mTIP(7–39) binds with lower affinity to the PTH2 receptor (14 nM versus 1.7 nM for the PTH1 receptor, measured in the presence of plasma for both receptors (Fig. 4B)). The binding activity in plasma was less effective at inhibiting $[^{125}\text{I}]\text{mTIP}(7-39)$ binding to the PTH2 receptor than the PTH1 receptor, at each time point tested. The pharmacological selectivity of the plasma binding activity is therefore similar to that of mTIP(7–39).

The plasma concentration of the binding activity was calculated using the percentage inhibition values (Fig. 4A) and the standard curve for mTIP(7–39) binding to the PTH1 receptor (Fig. 4B). This analysis assumes that the activity in plasma binds with the same affinity to the PTH1 receptor as mTIP(7–39). A dilution of plasma was used such that the level of inhibition was in the sensitive range of the mTIP(7–39) standard curve (between 10 and 90% inhibition). Fortunately, the level of inhibition at all time points was within this range using the same dilution of plasma (25 μl in a total assay volume of 125 μl). The time course of the binding activity was described by a bi-exponential decay function (Fig. 4C, Table 2). Most of the binding activity was reduced rapidly ($t_{1/2}$ of 2.5 min). A fraction of the activity (4%) was lost at a slower rate ($t_{1/2}$ of 20 min). These values are very similar to those for the disappearance of injected $[^{125}\text{I}]\text{mTIP}(7-39)$ bioactivity (Table 2).

4. Discussion

We have previously shown that bTIP(7–39) is a high-affinity PTH1 receptor antagonist in vitro [15]. The in vivo pharmacological properties of TIP(7–39) will likely

be evaluated in rodents. Therefore, we evaluated antagonist activity of TIP(7–39) on the rat PTH1 and PTH2 receptors, and investigated the plasma stability of the antagonist.

Recently, the sequence of mouse TIP39 was identified in genomic sequence [10]. mTIP(7–39) was synthesized and its antagonist activity compared with that of bTIP(7–39). On the rat PTH1 receptor mTIP(7–39) was 4.8-fold more potent than bTIP(7–39) in antagonizing PTHrP(1–34)-stimulated cAMP accumulation (Fig. 1A, Table 1). mTIP(7–39) differs from the bovine sequence at four positions in the C-terminal portion of the molecule; in mTIP39, Arg replaces His24 of the bovine sequence, Asp replaces Asn27, Gln replaces His 31 and Leu replaces Val35. The mechanism underlying the increase of affinity likely involves one or more of the substitutions increasing the affinity of TIP(7–39) for the large N-terminal extracellular domain of the receptor: N-terminally-truncated fragments of PTH have been shown to interact with this region of the receptor [20], and TIP(7–39) shares significant sequence homology [42] and a highly similar secondary structure [28] with PTH. mTIP(7–39) is also selective for the PTH1 receptor—it's antagonist potency on the rat PTH2 receptor was 21-fold lower (Fig. 1, Table 1).

The bioactivity of [125 I]mTIP(7–39) in plasma was first tested *in vitro*. Previously plasma has been shown to block the PTH1-receptor antagonist activity of a PTHrP(7–34) analogue, probably resulting from interaction with a plasma protein [22]. In this study pre-incubation with rat plasma at 37 °C slowly reduced [125 I]mTIP(7–39) binding to the PTH1 receptor ($t_{1/2}$ of 81 min), likely due to the action of serum proteases (Fig. 2). The bioactivity of mTIP(7–39) is therefore, relatively stable in rat plasma *in vitro*. We then tested the activity of both radiolabeled and unlabeled mTIP(7–39) *in vivo*, following intravenous administration into rats. Following injection of [125 I]mTIP(7–39), the radioactivity was cleared in a bi-phasic fashion, with a rapid rate of absorption ($t_{1/2}$ of 2.9 min) and a slower rate likely corresponding to elimination ($t_{1/2}$ of 120 min (Fig. 3A, Table 2). [125 I]mTIP(7–39) bioactivity (PTH1 receptor binding) was also cleared from plasma in a bi-phasic fashion (Fig. 3B). Most of the activity was removed rapidly (92%, $t_{1/2}$ = 3.6 min) but a significant fraction was cleared more slowly (8%, $t_{1/2}$ = 21 min). These kinetics were verified by the almost identical clearance parameters obtained for the binding activity of unlabeled mTIP(7–39) (Fig. 4C, Table 2).

Reversed-phase HPLC was used to determine the nature of the radioactive components in plasma taken from rats injected with [125 I]mTIP(7–39). Before loading onto the column, plasma was first treated to precipitate high-molecular weight material, which would otherwise have been precipitated by the organic phase during chromatography. TFA was initially used for this purpose (at 4% (v/v) final concentration) but a greater recovery of radioactivity was obtained using Bennett's cocktail [2]. With increasing time after administration, the peak corresponding to

intact [125 I]mTIP(7–39) decreased, with a $t_{1/2}$ of 3.4 min (Fig. 3C and D). The radioactivity in the column run-through increased over 2–12 min then reached a plateau (Fig. 3C). These observations suggest that the radioligand is degraded during the time course, explaining the reduction of bioactivity. However, the time course of removal of free intact [125 I]mTIP(7–39) was mono-exponential, in contrast to the bi-exponential removal of [125 I]mTIP(7–39) binding activity. The rate of the former was similar to the faster rate of the latter. The more slowly removed component of binding activity might therefore, be in the TFA- or Bennett's cocktail-precipitate (and so would not be detected by HPLC), possibly representing [125 I]mTIP(7–39) in complex with a plasma protein. This possibility requires further investigation.

In this study, we identified mTIP(7–39) as a high-affinity, selective antagonist of the rat PTH1 receptor. This high affinity should be useful for detecting any anti-hypercalcemic effect of the ligand in animal models of hypercalcemia. However, rapid clearance of mTIP(7–39) suggests that continuous administration will be required to detect an anti-hypercalcemic effect. A fraction of mTIP(7–39) binding activity was removed more slowly, which should aid in accomplishing effective plasma concentrations of the antagonist.

Acknowledgments

Funded by the National Institute of Mental Health Intramural Research Program. We thank Lidko Szalayova for help with cannulation.

References

- Akino A, Ohtsura A, Kanda K, Yasuda A, Yamamoto T, Akino Y, et al. Parathyroid hormone-related peptide is a potent tumor angiogenic factor. *Endocrinology* 2000;141:4313–6.
- Bennett HP, Hudson AM, McMartin C, Pardon GE. Use of octadecyl-silica for the extraction and purification of peptides in biological samples. Application to the identification of circulating metabolites of corticotropin-(1–24)-tetraacosapeptide and somatostatin *in vivo*. *Biochem J* 1977;168:9–13.
- Capparelli C, Kostenuik PJ, Morony S, Starnes C, Weimann B, Van G, et al. Osteoprotegerin prevents and reverses hypercalcemia in a murine model of humoral hypercalcemia of malignancy. *Cancer Res* 2000;60:783–7.
- Catlaiss C, Lieberherr M, Cros M, Gauville C, Graulet AM, Cotton J, et al. Parathyroid hormone-related peptide stimulates proliferation of highly tumorigenic human SV40-immortalized breast epithelial cells. *J Bone Miner Res* 2000;15:2129–39.
- Chorev M, Goldman ME, McKee RL, Roubini E, Levy JJ, Gay CT, et al. Modifications of position 12 in parathyroid hormone and parathyroid hormone related protein toward the design of highly potent antagonists. *Biochemistry* 1990;29:1580–6.
- Clark JA, Bonner TJ, Kim AS, Usdin TB. Multiple regions of ligand discrimination revealed by analysis of chimeric parathyroid hormone 2 (PTH2) and PTH/PTHrP-related peptide (PTHrP) receptors. *Mol Endocrinol* 1998;12:193–206.

- [7] Doppelt SH, Neer RM, Nussbaum SR, Federico P, Potts JT Jr. Rosenblatt M. Inhibition of the *in vivo* parathyroid hormone-mediated calcemic response in rats by a synthetic hormone antagonist. *Proc Natl Acad Sci USA* 1986;83:7557-60.
- [8] Dresner-Pollak R, Yang QM, Behar V, Nakamoto C, Chorev M, Rosenblatt M. Evaluation *in vivo* of a potent parathyroid hormone antagonist: [Nle⁸,Ile⁹-D-Trp¹²,Tyr³⁴]PTH(7-34)NH₂. *J Bone Miner Res* 1996;11:1061-5.
- [9] Fox J, Lowe SH, Petty BA, Nemeth EF. NPS R-568: a type II calcimimetic compound that acts on parathyroid cell calcium receptor of rats to reduce plasma levels of parathyroid hormone and calcium. *J Pharmacol Exp Ther* 1999;290:473-9.
- [10] Gould C, Usdin TB, Hoare SR. Receptors in rat and human parathyroid hormone (PTH) 2 receptors controlling receptor interaction with PTH and antagonist ligands. *J Pharmacol Exp Ther* 2001;299:678-90.
- [11] Grill V, Rankin W, Martin TJ. Parathyroid hormone-related protein (PTHrP) and hypercalcaemia. *Eur J Cancer* 1998;34:222-9.
- [12] Haq M, Krcmar R, Goltzman D, Rabbani SA. A vitamin D analogue (EB1089) inhibits parathyroid hormone-related peptide production and prevents the development of malignancy-associated hypercalcaemia *in vivo*. *J Clin Invest* 1993;91:2416-22.
- [13] Hoare SR, de Vries G, Usdin TB. Measurement of agonist and antagonist ligand-binding parameters at the human parathyroid hormone type 1 receptor: evaluation of receptor states and modulation by guanine nucleotide. *J Pharmacol Exp Ther* 1999;289:1323-33.
- [14] Hoare SR, Clark JA, Usdin TB. Molecular determinants of tuberoinfundibular peptide of 39 residues (TIP39) selectivity for the parathyroid hormone-2 (PTH2) receptor. N-terminal truncation of TIP39 reverses PTH2 receptor/PTH1 receptor binding selectivity. *J Biol Chem* 2000;275:27274-83.
- [15] Hoare SR, Usdin TB. Tuberoinfundibular peptide (7-39) [TIP(7-39)], a novel, selective, high-affinity antagonist for the parathyroid hormone-1 receptor with no detectable agonist activity. *J Pharmacol Exp Ther* 2000;295:761-70.
- [16] Hoare SRJ, Usdin TB. Quantitative cell membrane-based radioligand binding assays for parathyroid hormone receptors. *J Pharmacol Toxicol Meth* 1999;41:83-90.
- [17] Horieuchi N, Holick MF, Potts JT Jr, Rosenblatt M. A parathyroid hormone inhibitor *in vivo* design and biological evaluation of a hormone analog. *Science* 1983;220:1053-5.
- [18] Horieuchi N, Rosenblatt M. Evaluation of a parathyroid hormone antagonist in an *in vivo* multiparameter bioassay. *Am J Physiol* 1987;253:E187-92.
- [19] Jonsson KB, John MR, Geusens RC, Giardella TJ, Juppner H. Tuberoinfundibular peptide 39 binds to the parathyroid hormone (PTH)/PTHrP-related peptide receptor, but functions as an antagonist. *Endocrinology* 2001;142:704-9.
- [20] Juppner H, Schipani E, Bringham FR, McClure I, Keutmann IT, Potts JT Jr, et al. The extracellular amino-terminal region of the parathyroid hormone (PTH)/PTHrP-related peptide receptor determines the binding affinity for carboxyl-terminal fragments of PTH(1-34). *Endocrinology* 1994;134:879-84.
- [21] Juppner H, Abou-Samra AB, Freeman M, Kong XJ, Schipani E, Richards J, et al. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* 1991;254:1024-6.
- [22] Kukreja SCD, Anza JJ, Wimbiscus SA, Fisher JE, McKee RL, Caulfield MP, et al. Inactivation by plasma may be responsible for lack of efficacy of parathyroid hormone antagonists in hypercalcaemia of malignancy. *Endocrinology* 1994;134:2184-8.
- [23] Lam MH, Thomas RJ, Martin TJ, Gillespie MT, Jans DA. Nuclear and nucleolar localization of parathyroid hormone-related protein. *Immunol Cell Biol* 2000;78:395-402.
- [24] Liu B, Amizuka N, Goltzman D, Rabbani SA. Inhibition of processing of parathyroid hormone-related peptide by anti-sense tRNA: effect *in vitro* and *in vivo* on rat Leydig (11-500) tumor cells. *Int J Cancer* 1995;63:276-81.
- [25] McKee RL, Goldman ME, Caulfield MP, DeHaven PA, Levy JJ, Nutt RF, et al. The 7-34-fragment of human hypercalcaemia factor is a partial agonist/antagonist for parathyroid hormone-stimulated cAMP production. *Endocrinology* 1988;122:3008-10.
- [26] Nemeth EF. Fox J. Calcimimetic compounds: a direct approach to controlling plasma levels of parathyroid hormone in hyperparathyroidism. *Trends Endocrinol Metab* 1999;10:66-71.
- [27] Ogata IS. Parathyroid hormone-related protein as a potential target of therapy for cancer-associated morbidity. *Cancer* 2000;88:2909-11.
- [28] Piserchio A, Usdin T, Mierke DF. Structure of tuberoinfundibular peptide of 39 residues. *J Biol Chem* 2000;275:27284-90.
- [29] Potts JT Jr, Bringham FR, Giardella TJ, Nussbaum S, Segre G, Kronenberg HL. Parathyroid hormone: physiology, chemistry, biosynthesis, secretion, metabolism and mode of action. In: Williams RH, Wilson JD, Foster DW, editors. *Williams' Textbook of Endocrinology*. Philadelphia: Saunders, 1995. p. 920-66.
- [30] Rabbani SA, Glud J, Liu B, Goltzman D. Regulation *in vivo* of the growth of Leydig cell tumors by antisense ribonucleic acid for parathyroid hormone-related peptide. *Endocrinology* 1995;136:5416-22.
- [31] Rodan GA. Mechanisms of action of bisphosphonates. *Annu Rev Pharmacol Toxicol* 1998;38:375-88.
- [32] Rosen HN, Lim M, Garber J, Moreau S, Bhargava HN, Pallotta J, et al. The effect of PTH antagonist BIM-44002 on serum calcium and PTH levels in hypercalcemic hyperparathyroid patients. *Calcif Tissue Int* 1997;61:455-9.
- [33] Sato K, Yamakawa Y, Shizume K, Satoh T, Nohmi K, Demura H, et al. Passive immunization with anti-parathyroid hormone-related protein monoclonal antibody markedly prolongs survival time of hypercalcemic nude mice bearing transplanted human PTHrP-producing tumors. *J Bone Miner Res* 1993;8:849-60.
- [34] Schetz JA, Mayr CA, Taylor JE, Rosenblatt M, Chorev M, Davis TP. Distribution and pharmacokinetics of a potent peptide antagonist of parathyroid hormone and parathyroid hormone-related protein in the rat. *J Pharmacol Exp Ther* 1995;274:1456-62.
- [35] Schipani E, Karga H, Karaplis AC, Potts JT Jr, Kronenberg HM, Segre GV, et al. Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTHrP-related peptide receptor. *Endocrinology* 1993;132:2157-65.
- [36] Silverberg SJ, Bone HG, Marriott TB, Locker FG, Thys-Jacobs S, Dziedic G, et al. Short-term inhibition of parathyroid hormone secretion by a calcium-receptor agonist in patients with primary hyperparathyroidism. *New Engl J Med* 1997;337:1506-10.
- [37] Singer FR, Ritch PS, Lad TE, Ringenberg QS, Schiller JH, Recker RR, et al. Treatment of hypercalcemia of malignancy with intravenous edronate. A controlled, multicenter study. The hypercalcemia study group. *Arch Intern Med* 1991;151:471-6.
- [38] Strewler GJ. The physiology of parathyroid hormone-related protein. *New Engl J Med* 2000;342:177-85.
- [39] Strewler GJ. Medical approaches to primary hyperparathyroidism. *Endocrinol Metab Clin North Am* 2000;29:523-39.
- [40] Usdin TB. Evidence for a parathyroid hormone-2 receptor selective ligand in the hypothalamus. *Endocrinology* 1997;138:831-8.
- [41] Usdin TB, Hoare SRJ, Wang T, Mezey E, Kowalak JA. TIP39: a new neuropeptide and PTH2-receptor agonist from hypothalamus. *Nat Neurosci* 1999;2:941-3.
- [42] Usdin TB. The PTH2 receptor and TIP39: a new peptide-receptor system. *Trends Pharmacol Sci* 2000;21:128-30.



US006903194B1

(12) **United States Patent**
Sato et al.

(10) **Patent No.:** **US 6,903,194 B1**
(45) **Date of Patent:** **Jun. 7, 2005**

- (54) **ANTIBODY AGAINST HUMAN PARATHORMONE RELATED PEPTIDES**
- (75) **Inventors:** Koh Sato, Shizuoka (JP); Yuji Wakahara, Shizuoka (JP); Naohiro Yabuta, Shizuoka (JP)
- (73) **Assignee:** Chugai Seiyaku Kabushiki Kaisha, Tokyo (JP)
- (*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) **Appl. No.:** **09/269,332**

(22) **PCT Filed:** **Sep. 24, 1997**

(86) **PCT No.:** **PCT/JP97/03382**

§ 371 (c)(1),
(2), (4) **Date:** **Mar. 25, 1999**

(87) **PCT Pub. No.:** **WO98/13388**

PCT Pub. Date: **Apr. 2, 1998**

(30) **Foreign Application Priority Data**

Sep. 26, 1996 (JP) 8-255196
Jul. 24, 1997 (JP) 9-214168

- (51) **Int. Cl.7** **C07K 1/10; A61K 39/395**
- (52) **U.S. CL** **530/387.1; 424/130.1; 424/178.1**
- (58) **Field of Search** **530/387.1, 388.1, 530/388.24, 388.85, 350, 300; 424/130.1, 178.1, 133.1, 134.1, 135.1, 141.1, 156.1, 158.1; 514/2**

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,001,223 A 3/1991 Rosenblatt et al. 530/324
5,217,896 A 6/1993 Kramer et al. 435/240,27
5,626,845 A 5/1997 Yoneda et al. 424/145.1
5,849,695 A 12/1998 Cohen et al. 514/12
5,993,817 A 11/1999 Yoneda et al. 424/158.1

FOREIGN PATENT DOCUMENTS

EP 0 293 130 11/1988
EP 0293 158 11/1988
EP 0 449 405 10/1991
EP 0 811 383 12/1997
EP 0 878 201 A1 11/1998
EP 0 962 467 A1 12/1999
EP 1 004 313 A1 5/2000
EP 1 090 643 A1 4/2001
JP 2-207099 8/1990
JP 04-502408 5/1992
JP 04-228089 8/1992
JP 04-228089 A 8/1992
JP 4-228089 8/1992
JP 7-165790 6/1995
JP 7-316195 12/1995
JP 11-80025 3/1999
JP 11-222440 8/1999
JP 2000-080100 3/2000
WO WO 89/11297 11/1989

WO WO 89/11298 11/1989
WO WO 90/07861 7/1990
WO WO 91/16928 11/1991
WO WO 92/00753 1/1992
WO WO 92/17602 10/1992
WO WO 92/19759 11/1992
WO WO 93/13133 7/1993
WO WO 94/11523 5/1994
WO WO 96/03437 2/1996
WO WO 96/39184 2/1996
WO WO 96/22790 8/1996
WO WO 96/26737 9/1996
WO WO 96/33735 10/1996
WO WO 98/13388 4/1998
WO WO 98/51329 11/1998
WO WO 99/57139 11/1999

OTHER PUBLICATIONS

Wong YW et al. J. Immunol. Apr. 1, 1995; 154(7):3351-8.*
Kemp, B. et al., Parathyroid hormone-related protein of malignancy: active synthetic fragments, Science, 238:1568-1570 (1987).
Baba, PTH/PTHrP, *Clinical Calcium*, 5:97-101 (1995) [Eng. transl.].
Natsume et al., Binding assay and analysis of kinetic parameters by BIAcore biosensor, *Experimental Medicine*, 13:85-91 (1995) [Eng. transl.].
Shigeno, PTH/PTHrP, *Clinical Calcium*, 5:79-83 (1995) [Eng. transl.].
Ohtomo et al., Humanization of Mouse ONS-M21 Antibody with the Aid of Hybrid Variable Regions, *Molecular Immunology*, 32:407-416 (1995).
Coleman et al., Biochemical Mechanisms of Parathyroid Hormone Action, *The Parathyroids, Basic and Clinical Concepts*, 239-258 (1994).
Deftos et al., Utilization of a Potentially Universal Downstream Primer in the Rapid Identification and Characterization of V α Genes from Two New Human V α Gene Families, *Scandinavian Journal of Immunology*, 39:95-103 (1994).
Cuisinier et al., Mechanisms that generate human immunoglobulin diversity operate from the 8th week of gestation in fetal liver, *European Journal of Immunology*, 23:110-118 (1993).
Sato et al., Passive Immunization with Anti-Parathyroid Hormone-Related Protein Monoclonal Antibody Markedly Prolongs Survival Time of Hypercalcemic Nude Mice Bearing Transplanted Human PTHrP-Producing Tumors, *Journal of Bone and Mineral Research*, 8:849-860 (1993).

(Continued)

Primary Examiner—G. Nickol

Assistant Examiner—C. Yaen

(74) *Attorney, Agent, or Firm*—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

(57) **ABSTRACT**

Disclosed are an antibody against human para-thyroid-hormone-related protein, a DNA coding for the antibody, a recombinant vector containing the DNA, a transformant transformed with the recombinant vector, a method for preparation of the antibody, and uses of the antibody.

US 6,903,194 B1

Page 2

OTHER PUBLICATIONS

- Sato et al., Reshaping a Human Antibody to Inhibit the Interleukin 6-dependent Tumor Cell Growth, *Cancer Research*, 53:851-856 (1993).
- Abou-Samra et al., Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: A single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium, *Proceedings of the National Academy of Sciences*, 89:2732-2736 (1992).
- Burtis, Parathyroid Hormone-Related Protein: Structure, Function, and Measurement, *Clinical Chemistry*, 38:2171-2183 (1992).
- Carter et al., Humanization of an anti-p18^{HER2} antibody for human cancer therapy, *Proceedings of the National Academy of Sciences*, 89:4285-4289 (1992).
- Co et al., Chimeric and Humanized Antibodies with Specificity for the CD33 Antigen, *The Journal of Immunology*, 148:1149-1154 (1992).
- Co et al., Humanized antibodies for antiviral therapy, *Proceedings of the National Academy of Sciences*, 88:2869-2873 (1991).
- Gorman et al., Reshaping a therapeutic CD4 antibody, *Proceedings of the National Academy of Sciences*, 88:4181-4185 (1991).
- Jones et al., Rapid PCR-Cloning of Full-Length Mouse Immunoglobulin Variable Regions, *BioTechnology*, 9:88-89 (1991).
- Jüppner et al., A G Protein-Linked Receptor for Parathyroid Hormone and Parathyroid Hormone-Related Peptide, *Science*, 254:1024-1026 (1991).
- Karlsson et al., Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system, *Journal of Immunological Methods*, 145:229-240 (1991).
- Kettleborough et al., Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation, *Protein Engineering*, 4:773-783 (1991).
- Maeda et al., Construction of reshaped human antibodies with HIV-neutralizing activity, *Human Antibodies and Hybridomas*, 2:124-134 (1991).
- Tempest et al., Reshaping a Human Monoclonal Antibody to Inhibit Human Respiratory Syncytial Virus Infection in vivo, *BioTechnology*, 9:266-271 (1991).
- Mizushima et al., pEF-BOS, a powerful mammalian expression vector, *Nucleic Acids Research*, 18:5322 (1990).
- Stewart et al., Clinical Review 16: Parathyroid Hormone-Related Proteins: Coming of Age in the 1990s, *The Journal of Clinical Endocrinology & Metabolism*, 71:1410-1414 (1990).
- Belyavsky et al., PCR-based cDNA library construction: general cDNA libraries at the level of a few cells, *Nucleic Acids Research*, 17:2919-2933 (1989).
- LoBuglio et al., Mouse/human chimeric monoclonal antibody in man: Kinetics and immune response, *Proceedings of the National Academy of Sciences*, 86:4220-4224 (1989).
- Frohman et al., Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer, *Proceedings of the National Academy of Sciences*, 85:8998-9002 (1988).
- Kukreja et al., Antibodies to Parathyroid Hormone-related Protein Lower Serum Calcium in Adhymic Mouse Models of Malignancy-associated Hypercalcemia Due to Human Tumors, *The Journal of Clinical Investigation*, 82:1798-1802 (1988).
- Riechmann et al., Reshaping human antibodies for therapy, *Nature*, 332:323-327 (1988).
- Verhoeyen et al., Reshaping Human Antibodies: Grafting an Antilysozyme Activity, *Science*, 239:1534-1536 (1988).
- Chomczynski et al., Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction, *Analytical Biochemistry*, 162:156-159 (1987).
- Chothia, Canonical Structures for the Hypervariable Regions of Immunoglobulins, *Journal of Molecular Biology*, 196:901-917 (1987).
- Dariavach et al., Human immunoglobulin C δ gene encodes the Kern*Oz λ chain and C δ 4 and C δ 5 are pseudogenes, *Proceedings of the National Academy of Sciences*, 84:9074-9078 (1987).
- Kemp et al., Parathyroid Hormone-Related Protein of Malignancy: Active Synthetic Fragments, *Science*, 238:1568-1570 (1987).
- Kozak, At Least Six Nucleotides Preceding the AUG Initiator Codon Enhance Translation in Mammalian Cells, *Journal of Molecular Biology*, 196:947-950 (1987).
- Moseley et al., Parathyroid hormone-related protein purified from a human lung cancer cell line, *Proceedings of the National Academy of Sciences*, 84: 5048-5052 (1987).
- Sato et al., A highly sensitive bioassay for PTH using ROS 17/2.8 subclonal cells, *Acta Endocrinologica*, 116:113-120 (1987).
- Suva et al., A Parathyroid Hormone-Related Protein Implicated in Malignant Hypercalcemia: Cloning and Expression, *Science*, 237:893-896 (1987).
- Takahashi et al., Structure of Human Immunoglobulin Gamma Genes: Implications for Evolution of a Gene Family, *Cell*, 29:671-679 (1982).
- de St. Groth et al., Production of Monoclonal Antibodies: Strategy and Tactics, *Journal of Immunological Methods*, 35:1-21 (1980).
- Chingwin et al., Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonuclease, *Biochemistry*, 18:5294-5299 (1979).
- Galfre et al., Rat + rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG, *Nature*, 277: 131-133 (1979).
- Kearney et al., A New Mouse Myeloma Cell Line That Has Lost Immunoglobulin Expression But Permits The Construction of Antibody-Secreting Hybrid Cell Lines, *The Journal of Immunology*, 123:1548-1550 (1979).
- Mulligan et al., Synthesis of rabbit β -globin in cultured monkey kidney cells following infection with a SV40 β -globin recombinant genome, *Nature*, 277:108-114 (1979).
- Shulman et al., A better cell line making hybridomas secreting specific antibodies, *Nature*, 276:269-270 (1978).
- Trowbridge, Interspecies Spleen-Myeloma Hybrid Producing Monoclonal Antibodies Against Mouse Lymphocyte Surface Glycoprotein, T200, *The Journal of Experimental Medicine*, 148:313-323 (1978).
- Yelton et al., Fusion of Mouse Myeloma and Spleen Cells, *Lymphocyte Hybridomas, Second Workshop on "Functional Properties of Tumors of T and B Lymphocytes," Sponsored by the National Cancer Institute (NIH) 1-7 (1978).*

US 6,903,194 B1

Page 3

- Köhler et al., Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion, *European Journal of Immunology*, 6:511-519 (1976).
- Margulies et al., Somatic Cell Hybridization of Mouse Myeloma Cells, *Cell*, 8:405-415 (1976).
- Roe et al., A Photometric Method for the Determination of Inulin in Plasma and the Urine, *The Journal of Biological Chemistry*, 173:839-845 (1949).
- C. Queen, et al., "A humanized antibody that binds to the interleukin 2 receptor," *Proc. National Academy Science USA*, 86:10029-10033 (Dec. 1989).
- Mountain A, et al., Engineering antibodies for therapy (Article), *Biotechnol Genet Eng Rev* 10:1-142 (1992).
- Mountain A, et al., Engineering antibodies for therapy (Abstract only), *Biotechnol Genet Eng Rev* 10:1-142 (1992).
- Abou-Samra et al., Expression Cloning of a Common Receptor for Parathyroid Hormone and Parathyroid Hormone-Related Peptide from Rat Osteoblast-Like Cells: A Single Receptor Stimulates Intracellular Accumulation of Both cAMP and Inositol Triphosphates and Increases Intracellular Free Calcium, *Proceedings of the National Academy of Sciences*, 89:2732-2736 (1992).
- Baba, PTH/PTHrP, *Clinical Calcium*, 5:97-101 (1995) (English Translation).
- Beck, S.A., et al., Lipolytic Factor Associated with Murine and Human Cancer Cachexia, *Journal of the National Cancer Institute*, 82:1922-1926 (1990).
- Belyavsky et al., PCR-Based cDNA Library Construction: General cDNA Libraries at the Level of a Few Cells, *Nucleic Acids Research* 17:2919-2933 (1989).
- Benet et al., "Pharmacokinetics: The Dynamics of Drug Absorption, Distribution, and Elimination," In: *The Pharmacological Basis of Therapeutics*, 8th edition, pp. 3-32 (1990).
- Burtis, Parathyroid Hormone-Related Protein: Structure, Function, and Measurement, *Clinical Chemistry*, 38:2171-2183 (1992).
- Carter et al. Humanization of an Anti-p185^{HER2} Antibody for Human Cancer Therapy, *Proceedings of the National Academy of Sciences*, 89:4285-4289 (1992).
- Chirgwin et al., Isolation of Biologically Active Ribonucleic Acid From Sources Enriched in Ribonuclease, *Biochemistry*, 18:5294-5299 (1979).
- Chomczynski et al., Single-Step Method of RNA Isolation By Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction, *Analytical Biochemistry*, 162:156-159 (1987).
- Chothia, Canonical Structures for the Hypervariable Regions of Immunoglobulins, *Journal of Molecular Biology*, 196:901-917 (1987).
- Co et al., Chimeric and Humanized Antibodies with Specificity for the CD33 Antigen, *The Journal of Immunology*, 148:1149-1154 (1992).
- Carter et al (PNAS USA 1992 May; 89:4285-4289).*
- Co et al., Humanized Antibodies for Antiviral Therapy, *Proceedings of the National Academy of Sciences*, 88:2869-2873 (1991).
- Coleman et al., Biochemical Mechanisms of Parathyroid Hormone Action, *The Parathyroids, Basic and Clinical Concepts*, 239-258 (1994).
- Cuisinier et al., Mechanisms That Generate Human Immunoglobulin Diversity Operate From the 8th Week of Gestation in Fetal Liver, *European Journal of Immunology*, 23:110-118 (1993).
- Dariavach et al., Human Immunoglobulin C₁6 Gene Encodes the Kern OZ λ Chain and C₃4 and C₃5 as Pseudogenes, *Proceedings of the National Academy of Sciences*, 84:9074-9078 (1987).
- Deflios et al., Utilization of a Potentially Universal Downstream Primer in the Rapid Identification and Characterization of V λ Genes From Two New Human V λ Families, *Scandinavian Journal of Immunology*, 39:95-103 (1994).
- de St. Groth, et al., Production of Monoclonal Antibodies: Strategy and Tactics, *Journal of Immunological Methods*, 35:1-21 (1980).
- Dworkin, et al., Dietary Intake in Patients with Acquired Immunodeficiency Syndrome (AIDS), Patients with AIDS-Related Complex, and Serologically Positive Human Immunodeficiency Virus Patients: Correlations with Nutritional Status, *Journal of Parenteral and Enteral Nutrition*, 14:605-609 (1990).
- Farmer et al., Speculations on the Design of Nonpeptide Peptidomimetics, *TIIPS*, 4:362-365, (1982).
- Frohman et al., Rapid Production of Full-Length cDNAs From Rare Transcripts; Amplification Using a Single Gene-Specific Oligonucleotide Primer, *Proceedings of the National Academy of Sciences*, 85:8998-9002 (1988).
- Gallfre et al., Rat x Rat Hybrid Myelomas and A Monoclonal Anti-Fd Portion of Mouse IgG, *Nature*, 277:131-133 (1979).
- Gorman et al., Reshaping a Therapeutic CD4 Antibody, *Proceeding of the National Academy of Sciences*, 88:4181-4185 (1991).
- Hammond, M., et al., Respiratory Muscle Strength in Congestive Heart Failure, *Chest*, 98:1091-1094 (1990).
- Hardman, J.G. et al. (ed.), Goodman & Gilman's, "The Pharmacological Basis of Therapeutics", Section XIII, Hormones and Hormone Antagonists, McGraw-Hill Co. (USA) 9th ed., at 1528-1529, (1995).
- Hardman, J.G., et al., (ed.), "Agents Affecting Calcification and Bone Turnover" in Goodman and Gilman's "The Pharmacological Basis of Therapeutics", McGraw-Hill Co. (USA) 9th ed., at 1523-1524 (1995).
- Ikeda, K., "Molecular Biology of Parathyroid Hormone-Related Peptide", *Nihon Rinsho*, 53:37-45 (1995).
- Ikeda, "Development of Novel Endocrinotherapy Targeting Cancer and Paraneoplastic Syndromes," Progress in Clinical Pharmacology, 16:155-161 (1995) (English Abstract).
- Jones et al., Rapid PCR-Cloning of Full-Length Mouse Immunoglobulin Variable Regions, *BioTechnology*, 9:88-89 (1991).
- Jüppner et al., A G Protein-Linked Receptor for Parathyroid Hormone and Parathyroid Hormone-Related Peptide, *Science*, 254:1024-1026 (1991).
- Kaji, H. et al., "Role of Dual Signal Transduction Systems in the Stimulation of Bone Resorption by Parathyroid Hormone-Related Peptide, The Direct Involvement of cAMP-Dependent Protein Kinase," *Horm. Metab. Res.* 25:421-424 (1993).
- Kajimura, N., et al., Toxohormones Responsible for Cancer Cachexia Syndrome in Nude Mice Bearing Human Cancer Cell Lines, *Cancer Chemother Pharmacol*, 38:S48-S52 (1996).
- Karlsson et al., Kinetic Analysis of Monoclonal Antibody-Antigen Interactions with a New Biosensor Based Analytical System, *Journal of Immunological Methods*, 145:229-240 (1991).

US 6,903,194 B1

Page 4

- Kato, A., et al., "Incisor Change Induced by Excessive PTHrP in Rats," Abstracts of 16th Meeting of Japanese Society of Toxicologic Pathology, p. 17 (2000) (English Translation).
- Kearney et al., A New Mouse Myeloma Cell Line That Has Lost Immunoglobulin Expression But Permits the Construction of Antibody-Secreting Hybrid Cell Lines, *The Journal of Immunology*, 123:1548-1550 (1979).
- Kemp et al., Parathyroid Hormone-Related Protein of Malignancy: Active Synthetic Fragments, *Science* 238:1568-1570 (1987).
- Kettleborough et al., Humanization of a Mouse Monoclonal Antibody by CDR-Grafting: The Importance of Framework Residues on Loop Conformation, *Protein Engineering*, 4:773-738 (1991).
- Köhler et al., Derivation of Specific Antibody-Producing Tissue Culture and Tumor Lines by Cell Fusion, *European Journal of Immunology*, 6:511-519 (1976).
- Kozak, At Least Six Nucleotides Preceding the AUG Initiator Condon Enhance Translation in Mammalian Cells, *Journal of Molecular Biology*, 196:947-950 (1987).
- Kukreja et al., Tumor Resection and Antibodies to Parathyroid Hormone-Related Protein Cause Similar Changes on Bone Histomorphometry in Hypercalcemia of Cancer, *Endocrinology* 127(1):305-310 (1990).
- Kukreja et al., Antibodies to Parathyroid Hormone-Related Protein Lower Serum Calcium in Athymic Mouse Models of Malignancy-Associated Hypercalcemia Due to Human Tumors, *The Journal of Clinical Investigation*, 82:1798-1802 (1988).
- Liu, J.G. et al., "Developmental Role of PHTrP in Murine Molars," *European Journal Oral Sciences* 106 (suppl 1):143-146 (1998).
- LoBuglio et al., Mouse/Human Chimeric Monoclonal Antibody in Man; Kinetics and Immune Response, *Proceedings of the National Academy of Sciences*, 86:4220-4224 (1989).
- Lundgren et al., "Parathyroid Hormone (1-34) Receptor-Binding and Second-Messenger Response in Rat Incisor Odontoblasts", *Calcif. Tiss. Int.*, 62:255-259 (1998).
- Maeda et al., Construction of Reshaped Human Antibodies with HIV-Neutralizing Activity, *Human Antibodies and Hybridomas*, 2:124-134 (1991).
- Margulies et al., Somatic Cell Hybridization of Mouse Myeloma Cells, *Cell*, 8:405-415 (1976).
- Marosi, C., et al., Fatal Encephalitis in a Patient with Chronic Graft-Versus Host Disease, *Bone Marrow Transplantation*, 6:53-57 (1990).
- Mizushima et al., pEFBOS, A Powerful Mammalian Expression Vector, *Nucleic Acids Research*, 18:5322 (1990).
- Morimoto, PTH/PTHrP, *Clinical Calcium* 5(12):50-54 (1995) (English Translation).
- Moseley et al., Parathyroid Hormone-Related Protein Purified from A Human Lung Cancer Cell Line, *Proceedings of the National Academy of Sciences*, 84:5048-5052 (1987).
- Mountain A. et al., "Engineering Antibodies for Therapy," In: *Biotechnol Genet Eng Rev.* 10:1-142 (1992).
- Muller, J.M., et al., Überwachung und Handhabung von Zentrainervosen und Intestinalen System zur Behandlung der Tumorkachexie, *Langenbecks Arch Chir Suppl II*, p. 261-265 (1990).
- Mulligan et al., Synthesis of Rabbit β -globin in Cultured Monkey Kidney Cells Following Infection with a SV40 β -globin Recombinant Genome, *Nature*, 277:108-114 (1979).
- Natsume et al., Binding Assay and Analysis of Kinetic Parameters by Bialcore Biosensor, *Experimental Medicine*, 13:85-91 (1995) (English Translation).
- Ogata, E., "Parathyroid Hormone-Related Protein as a Potential Target of Therapy for Cancer-Associated Morbidity", *Cancer*, 88:2902-2911 (2000).
- Ohtoma et al., Humanization of Mouse ONS-M21 Antibody with the Aid of Hybrid Variable Regions, *Molecular Immunology* 32:407-416 (1995).
- Olstad, O.K., et al., Expression and Characterization of a Recombinant Human Parathyroid Partial Agonist with Antagonistic Properties: Gly-hPTH(-1 \rightarrow +84), *Peptides*, 16:1031-1037(1995).
- Palmieri et al., "Muscle Calcium Accumulation in Muscular Dystrophy," *Intracell. Calcium Regul., Proc. Int. Symp.*, pp. 335-347 (1986).
- Philbrick, et al., "Parathyroid Hormone-Related Protein is Required for Tooth Eruption", *Proc. Natl. Acad. Sci. USA*, 95:11846-11851 (1998).
- Queen, C., et al., "A Humanized Antibody that Binds to the Interleukin 2 Receptor," *Proc. National Academy of Science USA*, 86:10029-10033(1989).
- Riechmann et al., Reshaping Human Antibodies for Therapy, *Nature*, 332:323-327 (1988).
- Roe et al., A Photometric Method for the Determination of Insulin in Plasma and Urine, *Journal of Biological Chemistry*, 173:839-845 (1949).
- Rosen et al., "The effect of PTH Antagonist BIM-44002 on Serum Calcium and PTH Levels in Hypercalcemic Hyperparathyroid Patients", *Calcified Tissue International*, 61:455-459(1997).
- Roubini, E. et al., Synthesis of Fully Active Biotinylated Analogues of Parathyroid Hormone and Parathyroid Hormone-Related Protein as Tools for the Characterization of Parathyroid Hormone Receptors, *Biochemistry*, 31:4026-4033 (1992).
- Sato et al., Passive Immunization with Anti-Parathyroid Hormone-Related Protein Monoclonal Antibody Markedly Prolongs Survival Time of Hypercalcemic Nude Mice Bearing Transplanted Human PTHrP-Producing Tumors, *Journal of Bone and Mineral Research*, 8:849-860 (1993).
- Sato et al., A Highly Sensitive Bioassay for PTH Using ROS 17/2.8 Subclonal Cells, *Acta Endocrinologica*, 116:113-120 (1987).
- Sato, Malignancy-associated Hypercalcemia: Pathogenesis and Treatment, *Journal to Tokyo Women's Medical College*, 58(9):939-946 (1988) (English Abstract).
- Saito et al., "Potential Involvement of PHTrP in Cancer Cachexia," *Journal of Cancer Research*, 90(Suppl.): Abstract No. 2195 (1999) (English Abstract).
- Shigeno, C., PTH/PTHrP Receptor, *Clinical Calcium*, 5(3):79-83 (1995) (English Translation).
- Shulman et al., A Better Cell Line for Making Hybridomas Secreting Specific Antibodies, *Nature*, 276:269-270 (1978).
- Stewart et al., Clinical Review 16: Parathyroid Hormone-Related Proteins: Coming of Age in the 1990s, *Journal of Clinical Endocrinology and Metabolism*, 71:1410-1414 (1990).
- Strewler, G.J., "The Physiology of Parathyroid Hormone-Related Protein," *The New England Journal of Medicine*, *Mechanisms of Disease* 342(3):177-185 (2000).
- Sumiya et al., Hypercalcemia With Malignant Tumor, *Saishin Igaku*, 46(2):315-324 (1991).

US 6,903,194 B1

Page 5

- Suva et al., A Parathyroid Hormone-Related Protein Implicated in Malignant Hypercalcemia: Cloning and Expression, *Science*, 237:893-896 (1987).
- Takahashi et al., Structure of Human Immunoglobulin Gamma Genes: Implications for Evolution of a Gene Family, *Cell*, 29:671-679 (1982).
- Takahashi et al., "Concentrations of Blood Parathyroid Hormone Related Protein (PTHrP) and Various Cytokines in Malignant Tumor Patients," *Record of the Japan Society of Clinical Biochemistry and Metabolism*, 35:107 (1998) (English Abstract).
- Tanaka, R., Triple Paraneoplastic Syndrome of Hypercalcemia, Leukocytosis and Cachexia in Two Human Tumor Xerografts in Nude Mice, *Japanese Journal of Clinical Oncology*, 26:88-94 (1996).
- Tempester et al., Reshaping A Human Monoclonal Antibody to Inhibit Human Respiratory Syncytial Virus infection in vivo, *BioTechnology*, 9:266-271 (1991).
- Tenorio et al., "An immunohistochemical Investigation of the Expression of Parathyroid Hormone Receptors in Rat Cementoblasts", *Archs Oral Biol.*, 41:299-305 (1996).
- Sato et al., Reshaping a Human Antibody to Inhibit the Interleukin 6-dependent Tumor Cell Growth, *Cancer Research*, 53:851-856 (1993).
- Tisdale, M.J., et al., Cancer Cachexia, *International Journal of Pancreatolology*, 7:141-150 (1990).
- Trowbridge, Interspecies Spleen-Myeloma Hybrid Producing Monoclonal Antibodies Against Mouse Lymphocyte Surface Glycoprotein, T200, *Journal of Experimental Medicine*, 148:313-323 (1978).
- Verhoeven et al., Reshaping Human Antibodies; Grafting an Antilysozyme Activity, *Science* 239:1534-1536 (1988).
- Weissglas, M. et al., Hypercalcemia and Cosecretion of Interleukin-6 and Parathyroid Hormone Related Peptide by a Human Renal Cell Carcinoma Implanted into Nude Mice, *The Journal of Urology* 153:854-857 (1995).
- Wong et al., Modulation of Antibody Affinity by an Engineered Amino Acid Substitution, *J. Immunol.* 154(7):3351-8 (1995).
- Yamamoto, S., et al., "Parathyroid Hormone-Related Peptide-(1-34) PTHrP-(134) Induces Vasopressin Release from the Rat Supraoptic Nucleus *in Vitro* through a Novel Receptor Distinct from a Type I or Type II PTH/PTHrP Receptor", *Endocrinology*, 138(5):2066-2072 (1997).
- Yelton et al., Fusion of Mouse Myeloma and Spleen Cells, Lymphocyte Hybridomas, Second Workshop on "Functional Properties of Tumors of T and B Lymphocytes," Sponsored by the National Cancer Institute (NIH) 1-7 (1978).
- Yoshida et al., "Study of Abnormal Calcium Level in Myotonic Dystrophy-Part II: with Respect to Nephrogenous Cyclic AMP and Immunoreactivity of Serum Parathyroid Hormone," *The Japanese Endocrine Society Endocrine Journal*, 64(7):539-547 (1988) (English Abstract).
- Zbigniew Zylicz et al., Metabolic Response to Enteral Food in Different Phases of Cancer Cachexia in Rats, *Oncology*, 47:87-97 (1990).

* cited by examiner

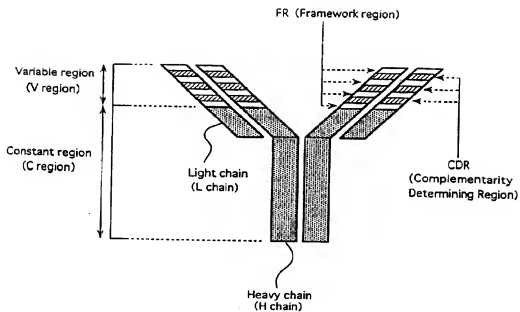
U.S. Patent

Jun. 7, 2005

Sheet 1 of 31

US 6,903,194 B1

FIG. 1



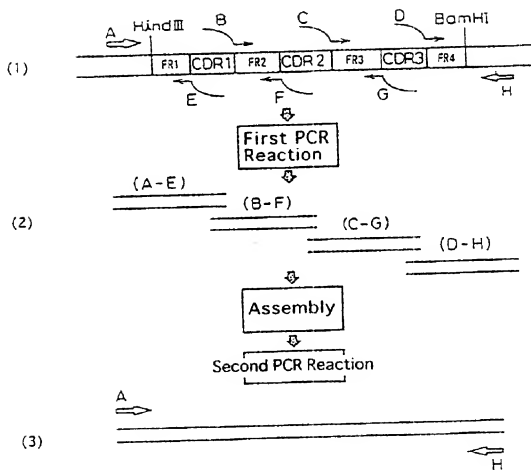
U.S. Patent

Jun. 7, 2005

Sheet 2 of 31

US 6,903,194 B1

FIG. 2



U.S. Patent

Jun. 7, 2005

Sheet 3 of 31

US 6,903,194 B1

v region					
FR1	FR2	FR3	FR4		
CDR1	CDR2	CDR3		Plasmid	Activity
H	H	m	m	h/mMBC1L (λ)	-
m	m	H	H	m/hMBC1L (λ)	+
H	m	m	m	hmmMBC1L (λ)	+
m	H	m	m	mhmMBC1L (λ)	-

H : FR of Human Antibody

m : FR of Mouse Antibody

FIG. 4

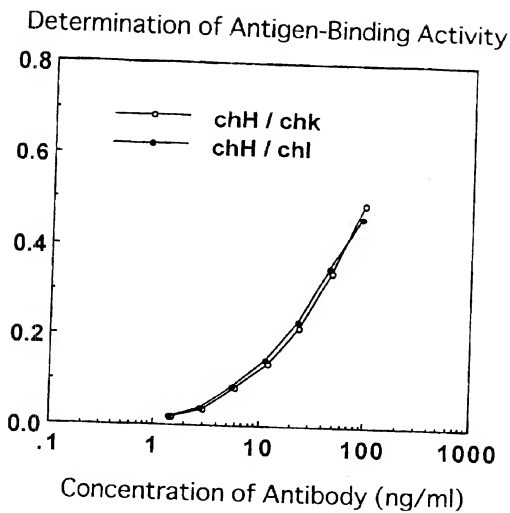


FIG. 5

Determination of Antigen-Binding Activity

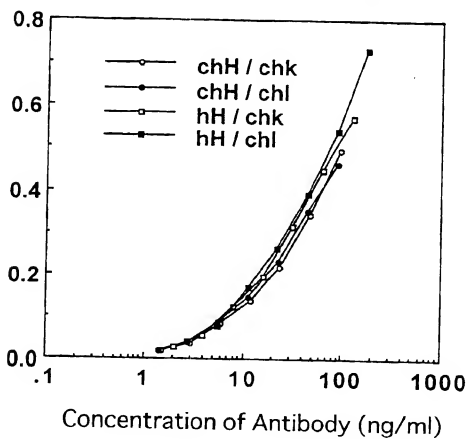


FIG. 6

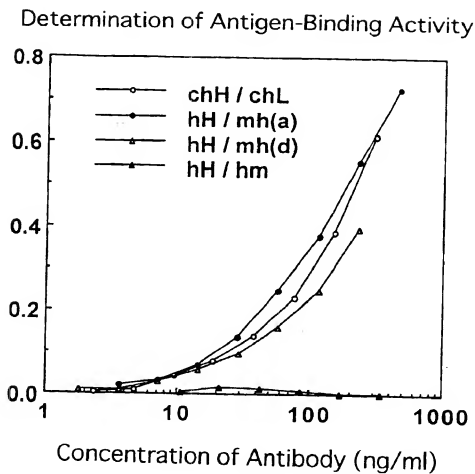


FIG. 7

Determination of Antigen-Binding Activity

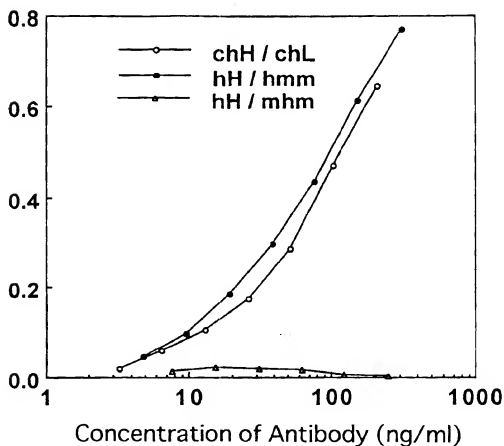


FIG.8

Determination of Antigen-Binding Activity

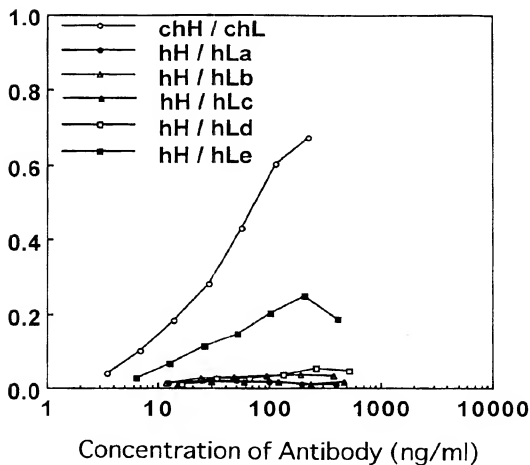


FIG. 9

Determination of Antigen-Binding Activity

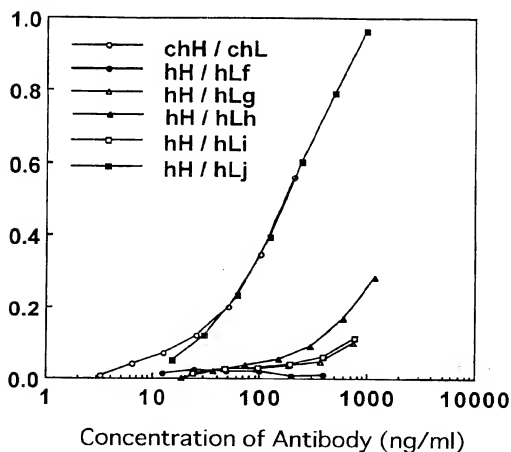


FIG. 10

Determination of Antigen-Binding Activity

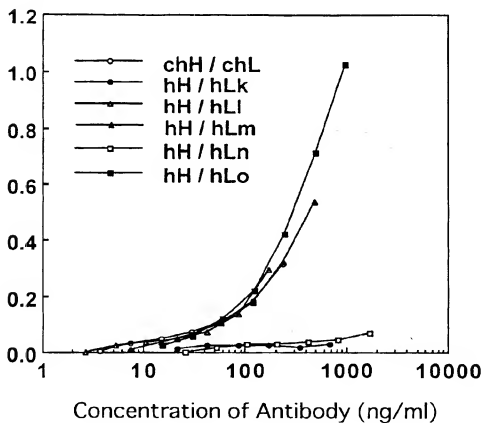
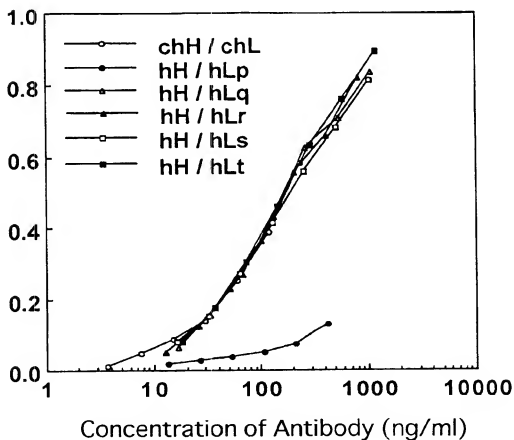


FIG. 11

Determination of Antigen-Binding Activity



U.S. Patent

Jun. 7, 2005

Sheet 12 of 31

US 6,903,194 B1

FIG. 12

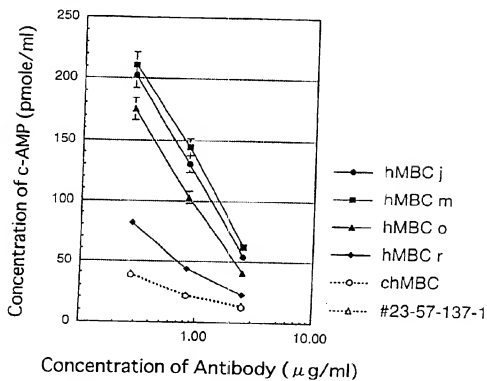
Neutralizing Activity of
Humanized anti-PTHrP (1-34) Antibody

FIG. 13

Neutralizing Activity of
Humanized anti-PTHrP (1-34) Antibody

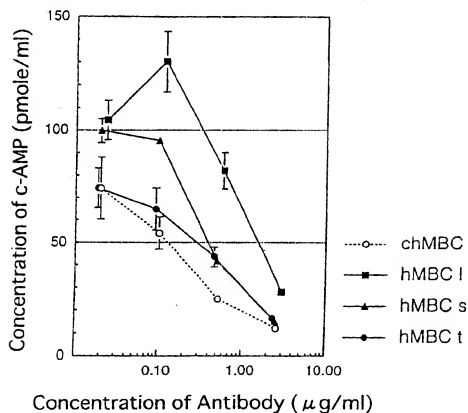
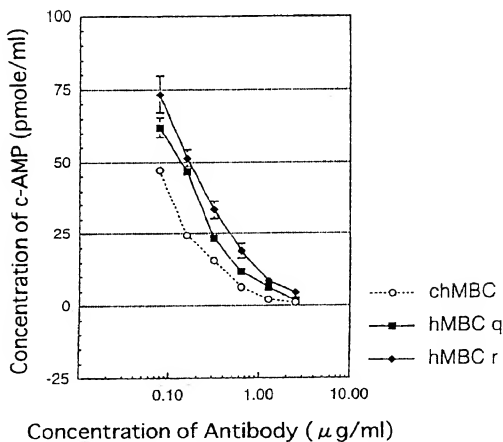


FIG. 14

Neutralizing Activity of
Humanized anti-PTHrP (1-34) Antibody



U.S. Patent

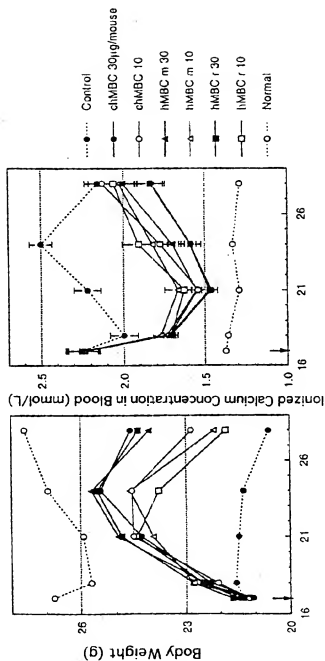
Jun. 7, 2005

Sheet 15 of 31

US 6,903,194 B1

FIG. 15

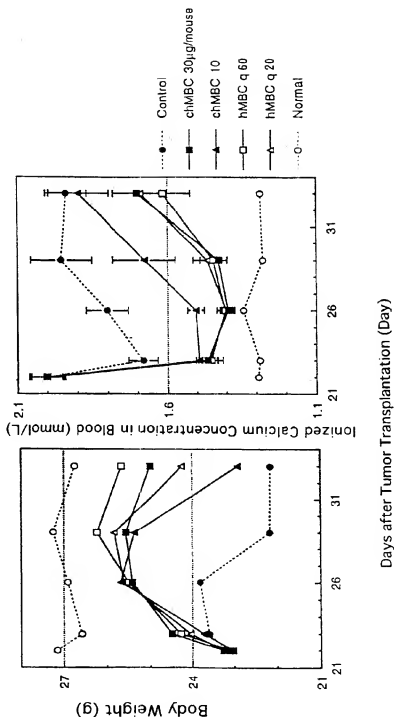
Effect of Chimeric Antibody and Humanized Antibody on Hypercalcemic Model Animal (Nude Mouse Carrying Human Pancreatic Cancer PAN-7)



Days after Tumor Transplantation (Day)

FIG. 16

Effect of Chimeric Antibody and Humanized Antibody on Hypercalcemic Model Animal (Nude Mouse Carrying Human Pancreatic Cancer PAN-7)



U.S. Patent

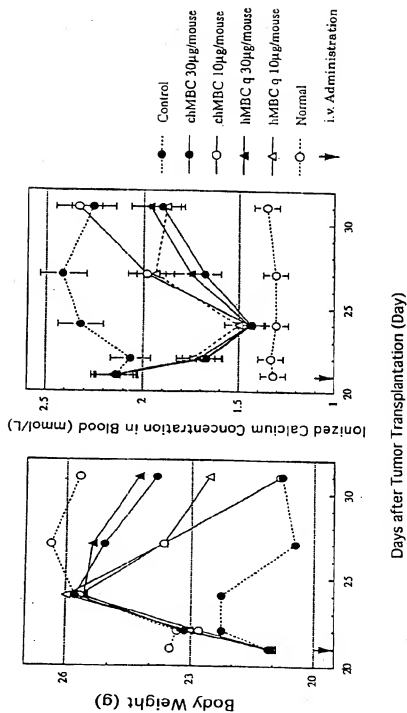
Jun. 7, 2005

Sheet 17 of 31

US 6,903,194 B1

FIG. 17

Effect of Chimeric Antibody and Humanized Antibody on Hypercalcemic Model Animal (Nude Mouse Carrying Human Pancreatic Cancer PAN-7)



U.S. Patent

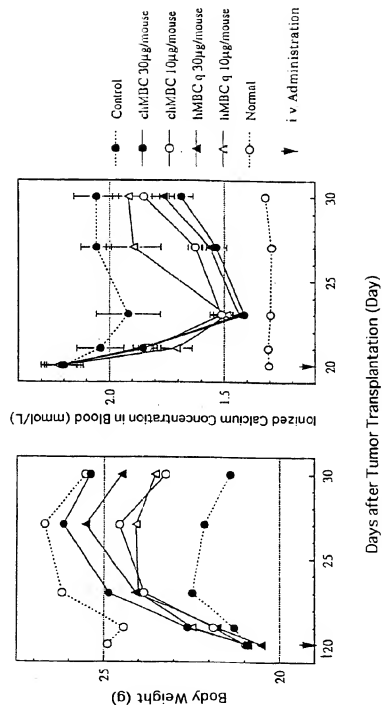
Jun. 7, 2005

Sheet 18 of 31

US 6,903,194 B1

FIG. 18

Effect of Chimeric Antibody and Humanized Antibody on Hypercalcemic Model Animal (Nude Mouse Carrying Human Lung Cancer LC-6-JCK)

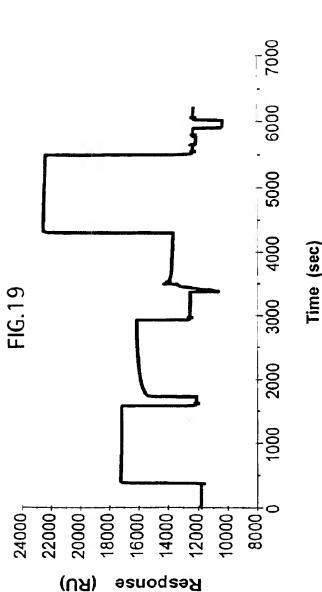


U.S. Patent

Jun. 7, 2005

Sheet 19 of 31

US 6,903,194 B1



Fc	Time	Window	AbsResp	SD	Slope	Baseline	RelResp	Id
2	368.5	5.0	11784.0	0.17	0.07	Yes	0	pre-NHS-EDC
2	1622	5.0	12157.3	2.29	-1.22	Yes	373.2	NHS-EDC-100ul
2	2966	5.0	12604.9	1.36	-0.71	No	447.6	PDEA-100ul
2	3530	5.0	14058.6	8.34	-4.45	No	1901.3	(1-34+C)5ug/ml-10ul-pH5.0
2	5545	5.0	12423.6	2.08	-1.10	No	266.3	Cys/NaCl-100ul
2	5804	5.0	12396.6	0.28	-0.13	No	239.3	Gly/HCl-10ul
2	6063	5.0	12383.6	0.13	0.00	No	226.4	10mM-HCl-10ul

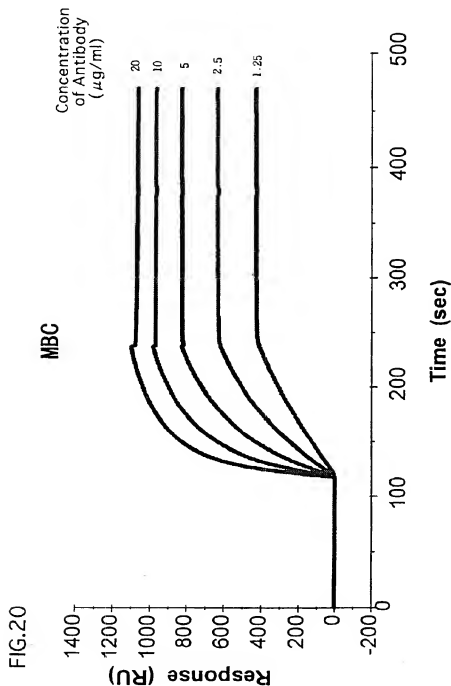
Sensorgram of Mobilization of PTHrP (1-34+C) onto Sensor Tip

U.S. Patent

Jun. 7, 2005

Sheet 20 of 31

US 6,903,194 B1



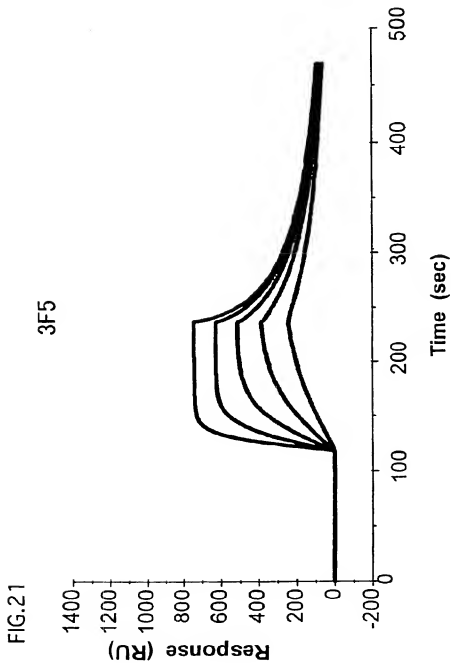
Overlaying of MBC Sensorgram

U.S. Patent

Jun. 7, 2005

Sheet 21 of 31

US 6,903,194 B1



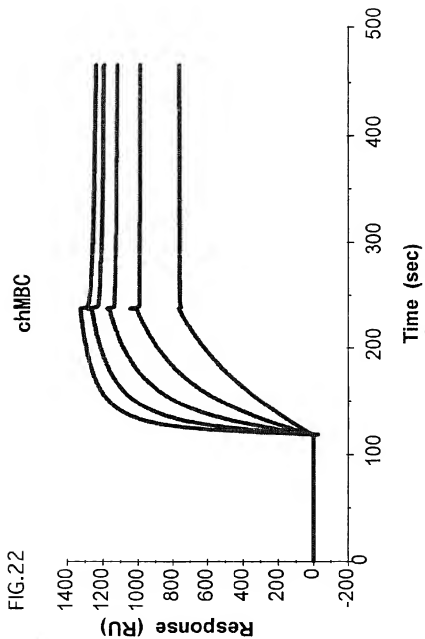
Overlaying of 3F5 Sensorgram

U.S. Patent

Jun. 7, 2005

Sheet 22 of 31

US 6,903,194 B1



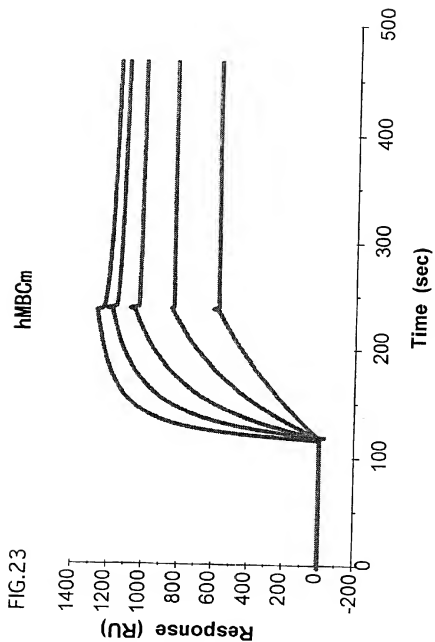
Overlaying of chMBC Sensorgram

U.S. Patent

Jun. 7, 2005

Sheet 23 of 31

US 6,903,194 B1



U.S. Patent

Jun. 7, 2005

Sheet 24 of 31

US 6,903,194 B1

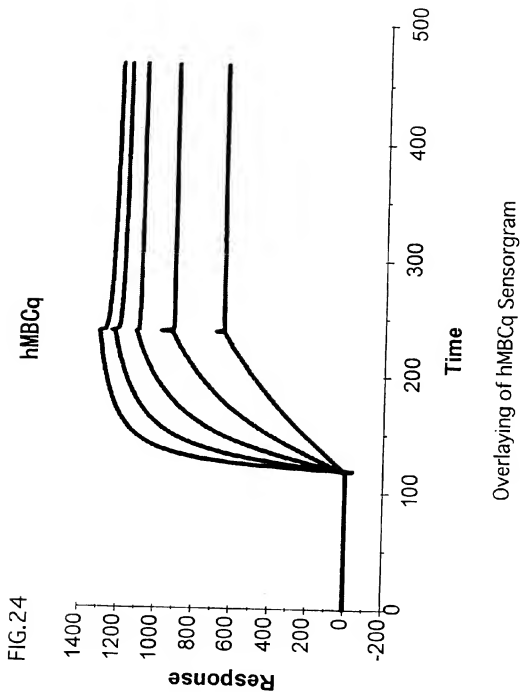


FIG.25

Effect on Fractional Excretion of Phosphate

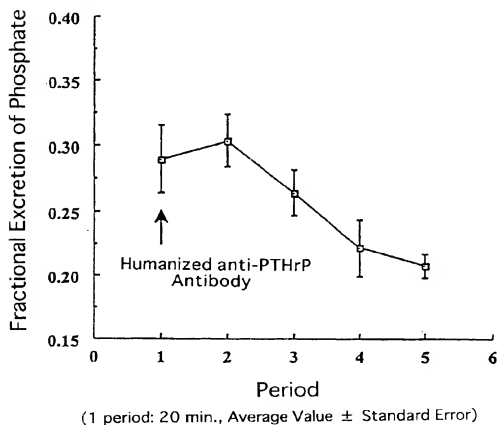
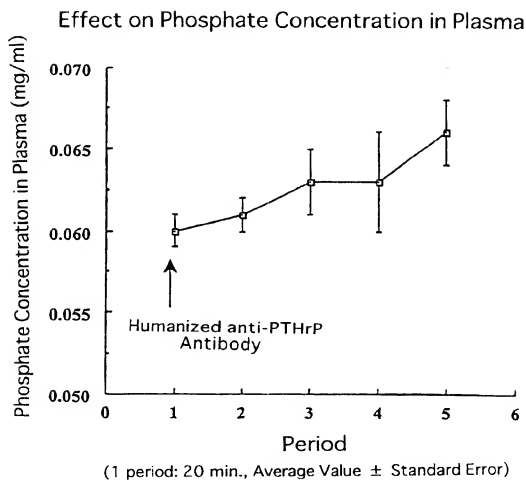


FIG.26



U.S. Patent

Jun. 7, 2005

Sheet 27 of 31

US 6,903,194 B1

FIG. 27



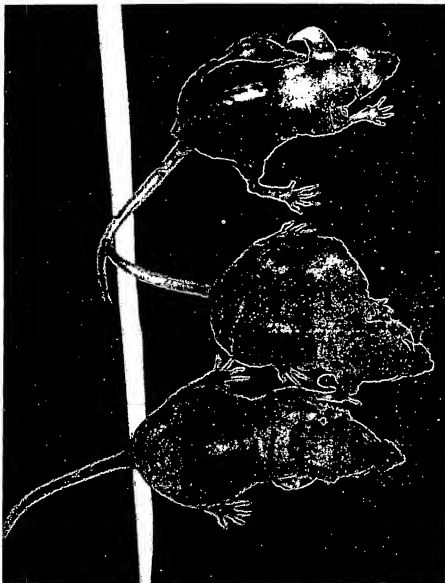
U.S. Patent

Jun. 7, 2005

Sheet 28 of 31

US 6,903,194 B1

FIG.28



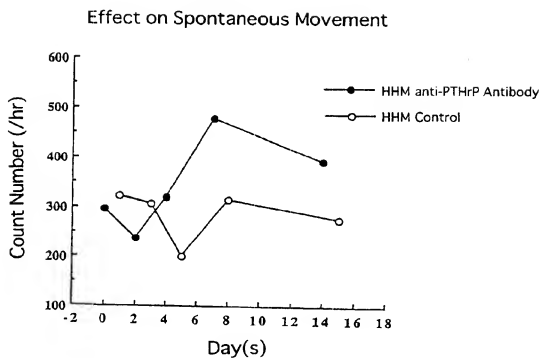
U.S. Patent

Jun. 7, 2005

Sheet 29 of 31

US 6,903,194 B1

FIG.29



U.S. Patent

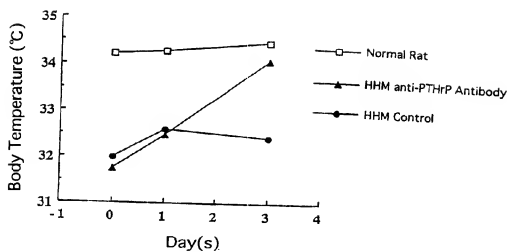
Jun. 7, 2005

Sheet 30 of 31

US 6,903,194 B1

FIG.30

Effect on Body Temperature



U.S. Patent

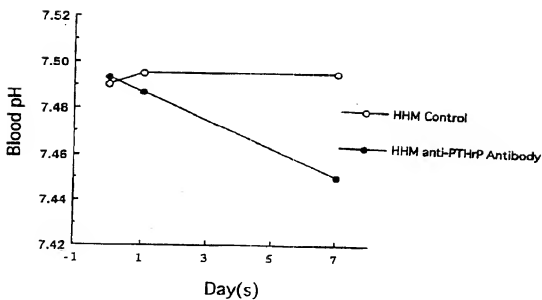
Jun. 7, 2005

Sheet 31 of 31

US 6,903,194 B1

FIG.31

Effect on Blood pH



US 6,903,194 B1

1

ANTIBODY AGAINST HUMAN PARATHORMONE RELATED PEPTIDES

TECHNICAL FIELD

The present invention relates to a human/mouse chimeric antibody comprising a variable region (V region) of a mouse monoclonal antibody against a parathyroid hormone related protein and a constant region (C region) of a human antibody, a humanized antibody in which complementarity determining regions of the light chain (L chain) and heavy chain (H chain) V regions of a mouse monoclonal antibody against a parathyroid hormone related protein (PTHrP) are grafted to a human antibody, the L and H chains of said antibody, as well as a polypeptide comprising the V region constituting the L or H chain of said antibody.

The present invention also relates to a DNA comprising a base sequence coding for the above mentioned antibody, particularly its V region, and a DNA coding for an L or H chain comprising the V region. Further, the present invention relates to a recombinant vector comprising said DNA and a host transformed with said vector.

Furthermore, the present invention relates to processes for preparing the chimeric and humanized antibodies against a PTHrP. Still further, the present invention relates to a pharmaceutical composition, and hypercalcemia-suppressing or hypophosphatemia-improving agent comprising the antibody against a PTHrP as an effective ingredient.

BACKGROUND OF THE INVENTION

Hypercalcemia associated with malignant tumor is a serious complicated symptom found in 5 to 20% of the whole patients suffering from malignant tumor and is considered to be a terminal symptom of malignant tumor since it certainly leads to death if it is left as it is. Control of hypercalcemia may greatly affect the prognosis and QOL (quality of life) of a patient; therefore, it will clinically play a significant role.

Generally, hypercalcemia in patients suffering from malignant tumor is roughly classified between HHM (humoral hypercalcemia of malignancy) based on tumor-producing humoral bone resorption factors and LOH (local osteolytic hypercalcemia) based on local action of tumor transferred or infiltrated to the bone. In HHM, it is believed that bone resorption or osteoclasts is promoted to increase the flow of calcium and produces hypercalcemia in cooperation with the reduced renal calcium-excreting ability (S. Wada and N. Nagata, *Internal Medicine*, 69, 644-648).

Hypercalcemia is considered to exhibit its symptoms when the concentration of calcium in the serum exceeds 12 mg/dl; as its symptoms, anorexia (inappetence), nausea and emesis (vomiting) are non-specifically observed at the early stage in patients suffering from malignant tumor. When hypercalcemia is worsened, the reduction of water-concentrating ability due to lesion of the renal distal tubules leads to hyperuricemia (polyuria) and anorexia and nausea will be accompanied with dehydration due to insufficient uptake of water.

As humoral factors causing HHM among the hypercalcemia associated with malignant tumor, Moseley, J. M. et al. found parathyroid hormone related protein (hereinafter referred to as "PTHrP") which are substances like parathyroid hormone (PTH): *Proc. Natl. Acad. Sci. USA* (1987) 84, 5048-5052.

2

Thereafter, a gene coding for PTHrP was isolated (Suva, L. J. et al., *Science* (1987) 237, 893) and it was elucidated from its analysis that there are three kinds of human PTHrPs having 139, 141 and 173 amino acids due to alternative splicing of the gene and that various fragments are present in the blood due to restricted degradation of PTHrP (1-139) having the whole structure: Baba, H., *Clinical Calcium* (1995) 5, 229-223. In PTHrP, 8 amino acids of the N-terminal 13 amino acids are identical with those in PTH and it is deduced that the amino acid site at position 14 to position 34 has a steric structure similar to PTH as well; thus, PTHrP and PTH bind to a common PTH/PTHrP receptor at least in the N-terminal region: Jueppner, H. et al., *Science* (1991) 254, 1024-1026; Abou-Samra, A.-B. et al., *Proc. Natl. Acad. Sci. USA* (1992) 89, 2732-2736.

PTHrP is reported to be produced in a variety of tumoral tissues and it has been elucidated that not only in tumors, PTHrP is also produced in various normal tissues of from fetuses to adults, including skin, central nervous system, uterus, placenta, lactating mammary gland, thyroid gland, parathyroid gland, adrenal gland, liver, kidney and urinary bladder: Burris, W. J., *Clin. Chem.* (1992) 38, 2171-2183; Stewart, A. F. & Broadus, A. E., *J. Clin. Endocrinol.* (1991) 71, 1410-1414. Further, PTHrP is considered to play an important role in the metabolic regulation of calcium which is maintained at a higher level in the fetal to newborn period than in the mother.

PTH/PTHrP receptors are known to be present mainly in the bone and kidney (C. Shigeno, *Clinical Calcium* (1995) 5, 355-359) and to activate plural intracellular signal transmission systems by binding of PTHrP to the receptors. One of them is adenylate cyclase and the other is phospholipase C. Activation of adenylate cyclase increases the concentration of intracellular cAMP to activate protein kinase A. Phospholipase C decomposes phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate and diacylglycerol. G-protein is involved in these signal transmission systems: Coleman, D. T. et al., *Biochemical mechanisms of parathyroid hormone action*. In: "The parathyroids" (Bilezikian, J. P. et al.), Raven Press, New York (1994) page 239.

Through these signal transmission systems, PTHrP causes hypercalcemia, hypophosphatemia, decrease of renal phosphate-reabsorbing ability, increase of renal cAMP-excretion and the like which are observed in HHM.

Thus, it has been elucidated that PTHrP is closely related to hypercalcemia associated with malignant tumor. In the treatment of hypercalcemia associated with malignant tumor, calcitonin, steroid agents, indomethacin, inorganic phosphate salts, bisphosphonates and the like are used, as well as fluid replacement. However, these agents may show reduction of their effects upon consecutive use, some serious side-effects, or slow expression of their pharmacological effects; accordingly, use of agents or drugs which have higher therapeutic effects and less side-effects is highly expected.

On the other hand, as a new attempt to treat hypercalcemia associated with malignant tumor, Kukreja, S. C. et al. reported that when a neutralizing antiserum against PTHrP was administered to athymic mice in which human lung or larynx cancer cells had been transplanted to generate hypercalcemia, the blood calcium concentration and urinary cAMP level were reduced: *J. Clin. Invest.* (1988) 82, 1798-1802. Kanji Sato et al. reported that when an antibody against PTHrP (1-34) was administered to nude mice to which a PTHrP-producing human tumor was transplanted,

US 6,903,194 B1

3

the hypercalcemia was reduced and the viable time period of the mice was greatly prolonged: J. bone & Mine. Res. (1993) 8, 849-860. Further, Japanese Patent Application Laid Open Publication No. 4-228089 discloses mouse/human chimeric antibodies against human PTHrP (1-34).

Mouse monoclonal antibodies are highly immunogenic (sometimes also referred to as "antigenic") in humans, which limits the medical therapeutic values of the mouse monoclonal antibodies in humans. For instance, a mouse antibody may be metabolized as a foreign matter when administered to a human; therefore, the half-life of the mouse antibody is relatively short in humans and its expected effects are not sufficiently exhibited. Further, human anti-mouse antibodies. (HAMA) raised against the administered mouse antibody may cause immune responses which are inconvenient and dangerous to patients, such as serum diseases and other allergic reactions. Accordingly, mouse monoclonal antibodies can not frequently be administered to humans.

In order to solve these problems, methods for reducing the immunogenicity of non-human derived antibodies, for example, mouse-derived monoclonal antibodies have been developed. One of these methods is to make a chimeric antibody in which the variable-region (V region) is derived from a mouse monoclonal antibody and the constant region (C region) is derived from an appropriate human antibody.

Since the resulting chimeric antibody has the intact variable region of the original mouse antibody, it can be expected that the chimeric antibody may bind to an antigen with the same specificity as the original mouse antibody. Further, such a chimeric antibody has a substantially reduced proportion of an amino acid sequence derived from a non-human animal; therefore, it is anticipated to have a lower immunogenicity as compared with the original mouse antibody. Although the chimeric antibody binds to its antigen equivalently with the original mouse monoclonal antibody while showing a lower immunogenicity, some immune responses to the mouse variable region may still be possibly generated: LoBuglio, A. F. et al., Proc. Natl. Acad. Sci. USA, 86, 4220-4224, 1989.

A second method for reducing the immunogenicity of mouse antibodies is still more complicated but expected to further greatly reduce the potential immunogenicity of the mouse antibodies. In this method, only the complementarity determining regions (CDRs) of the variable region of a mouse antibody are grafted to a human variable region to create a "reshaped" human variable region. If required, a partial amino acid sequence of a framework region (FR) supporting the CDRs in a variable region of a mouse antibody may be grafted to a human variable region in order to make the structure of CDRs in the reshaped human variable region closer to that of the original mouse antibody.

Then, these humanized, reshaped human variable regions are combined with human constant regions. In the finally reshaped, humanized antibody, the portions derived from non-human amino acid sequences are only CDRs and a very small part of FR. The CDRs are composed of a hypervariable amino acid sequence and these do not show any species-specific sequences. Therefore, a humanized antibody comprising mouse CDRs will no longer have any stronger immunogenicity than a naturally occurring human antibody containing human CDRs.

With respect to humanized antibodies, further reference should be made to Riechmann, L. et al., Nature, 332, 323-327, 1988; Verhoeve, M. et al., Science, 239, 1534-1536, 1988; Kettleborough, C. A. et al., Protein

4

Engng., 4, 773-783, 1991; Maeda, H. et al., Human Antibodies and Hybridoma, 2, 124-134, 1991; Gorman, S. D. et al., Proc. Natl. Acad. Sci. USA, 88, 4181-4185, 1991; Tempest, P. R. et al., Bio/Technology, 9, 266-271, 1991; Co, M. S. et al., Proc. Natl. Acad. Sci. USA, 88, 2869-2873, 1991; Carter, P. et al., Proc. Natl. Acad. Sci. USA, 89, 4285-4289, 1992; Co, M. S. et al., J. Immunol., 148, 1149-1154 1992; and Sato, K. et al., Cancer Res., 53, 851-856, 1993.

Although humanized antibodies are expected to be useful for therapeutic purposes as previously mentioned, no humanized antibody against PTHrP has been known nor suggested in the aforementioned references. Further, there is no standardized means generally applicable to any antibodies in the process for preparing humanized antibodies; various means and methods are necessary to make a humanized antibody exhibiting a sufficient binding, neutralizing activity to a specific antigen: see, for example, Sato, K. et al., Cancer Res., 53, 851-856, 1993.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide a human/mouse chimeric antibody comprising a variable region (V region) of a mouse monoclonal antibody against PTHrP and a constant region (C region) of a human antibody, a humanized antibody in which complementarity determining regions of V regions of the light chain (L chain) and heavy chain (H chain) of a mouse monoclonal antibody against PTHrP are grafted to a human antibody, the L and H chains of said antibody, as well as a polypeptide comprising the V region constituting the L or H chain of said antibody.

It is another object of the present invention to provide a DNA comprising a base sequence coding for the above mentioned antibody, particularly its V region, and a DNA coding for an L or H chain comprising a polypeptide comprising the V region. Still another object of the present invention is to provide a recombinant vector comprising said DNA and a host transformed with said vector. Further, an object of the present invention is to provide processes for preparing the chimeric and humanized antibodies against PTHrP. It is a still another object of the present invention to provide an antibody against PTHrP having a high neutralizing activity. A still further object of the present invention is to provide a pharmaceutical composition, and hypercalcemia-suppressing, hypophosphatemia-improving or alkalosis-improving agent comprising the antibody or humanized antibody against PTHrP as an effective ingredient.

As a result of the energetic study with a view to the above mentioned objects, the present inventors have successfully obtained an antibody in which the immunogenicity of mouse monoclonal antibodies against PTHrP is reduced in humans; thus, the present invention has been accomplished.

The present invention is directed to a chimeric L chain comprising an L chain C region of a human antibody and an L chain V region of a mouse monoclonal antibody against PTHrP. The L chain V region includes one comprising an amino acid sequence as shown in SEQ ID NO:45 and the L chain C region includes a C α region.

The present invention is also directed to a chimeric H chain comprising an H chain C region of a human antibody and an H chain V region of a mouse monoclonal antibody against PTHrP. The H chain V region includes one comprising an amino acid sequence as shown in SEQ ID NO:46 and the C region includes a C α region.

Further, the present invention is directed to a chimeric monoclonal antibody against PTHrP comprising said chimeric L chain and said chimeric H chain.

US 6,903,194 B1

5

Still further, the present invention includes a polypeptide comprising an L chain V region of a humanized antibody comprising framework regions 1 to 4 of an L chain V region of a human antibody and complementarity determining regions 1 to 3 of an L chain V region of a mouse monoclonal antibody against PTHrP. The complementarity determining regions 1 to 3 include those comprising amino acid sequences as shown in SEQ ID NOS: 59-61, respectively; the framework regions 1 to 3 include those derived from the framework regions 1 to 3 of human antibody HSU03868, respectively, and the framework region 4 includes one derived from the framework region 4 of human antibody S25755; or the framework regions 1 to 3 include those substantially identical with the framework regions 1 to 3 of human antibody HSU03868, respectively, and the framework region 4 includes one substantially identical with the framework region 4 of human antibody S25755.

The term "substantially identical" as used herein means that the framework regions of the human antibody used in a humanized antibody may have a deletion, replacement and/or addition of amino acid(s) required to form the complementarity determining regions of a mouse monoclonal antibody such that the humanized antibody should have an activity equivalent to that of the mouse monoclonal antibody.

Thus, the present invention is concerned with a polypeptide comprising an L chain V region of a humanized antibody wherein in the framework regions the 36th and 49th amino acids in accordance with Kabat's prescription (Kabat, E. A. et al., US Dept. Health and Human Services, US Government Printing Offices, 1991) are tyrosine and aspartic acid, respectively.

The present invention is also directed to a polypeptide comprising an L chain V region of a humanized antibody comprising an amino acid sequence as shown in any of SEQ ID NOS:48-51.

Further, the present invention is directed to a polypeptide comprising an L chain V region of a humanized antibody wherein the 45th and 87th amino acids in accordance with Kabat's prescription in the framework regions are lysine and isoleucine, respectively.

Still further, the present invention is directed to a polypeptide comprising an L chain V region of a humanized antibody comprising an amino acid sequence as shown in any of SEQ ID NOS: 52-55.

The present invention is further concerned with a polypeptide comprising an H chain V region of a humanized antibody comprising framework regions 1 to 4 of an H chain V region of a human antibody and complementarity determining regions 1 to 3 of an H chain V region of a mouse monoclonal antibody against a human PTHrP. The complementarity determining regions 1 to 3 include those comprising amino acid sequences as shown in SEQ ID NOS:62-64, respectively, the framework regions 1 to 4 include those derived from framework regions 1 to 4 of a human antibody belonging to human subgroup III (Human Subgroup III (HSG III), Kabat, E. A. et al., US Dept. Health and Human Services, US Government Printing Offices, 1991), more particularly those derived from the framework regions 1 to 4 of human antibody S31679, respectively, or those substantially identical with the framework regions 1 to 4 of human antibody S31679, respectively.

Also, the present invention is concerned with a polypeptide comprising an H chain V region of a humanized antibody comprising the amino acid sequence as shown in SEQ ID NO:56.

6

The present invention is also concerned with an L chain of a humanized antibody against a human PTHrP comprising a polypeptide comprising an L chain V region of said humanized antibody and a polypeptide comprising an L chain C region of a human antibody. The C region includes a C α region, the framework regions 1 to 3 include those substantially identical with the framework regions 1 to 3 of human antibody HSU03868, respectively, the framework region 4 includes one substantially identical with the framework region 4 of human antibody S25755, and the amino acid sequences of the complementarity determining regions 1 to 3 include those represented by SEQ ID NOS:59-61, respectively.

Further, the present invention is also concerned with an H chain of a humanized antibody against a human PTHrP comprising polypeptides comprising an H chain C region and H chain V region of said human antibody. The C region includes a C γ region, the framework regions 1 to 4 include those derived from the framework regions 1 to 4 derived from a human antibody belonging to HSGIII, and the complementarity determining regions 1 to 3 include those comprising the amino acid sequences as shown in SEQ ID NOS:62-64, respectively.

Still further, the present invention is also concerned with an anti-PTHrP antibody with a weak antigenicity and a high neutralizing activity. The PTHrP antibody includes a human antibody, a humanized antibody, a chimeric antibody and a primatized antibody, which may be utilized in the treatment of human diseases. The antibody has a low dissociation constant. Further, the antibody of the present invention has a high neutralizing activity due to its low dissociation constant and, therefore, can be utilized for the treatment of human diseases.

The antibody of the present invention has a dissociation constant of 1.86×10^{-7} [M] or less, a dissociation rate constant of 1.22×10^{-3} [1/Sec] or less, and an association rate constant of 6.55×10^4 [1/M.Sec] or more. These constants may be measured by Scatchard analysis using RI labeled ligands or Surface plasmon resonance sensor.

The present invention is further directed to a DNA comprising a base sequence coding for an L chain V region or H chain V region of a mouse monoclonal antibody against a human PTHrP. The L chain V region and H chain V region include those comprising the amino acid sequence as shown in SEQ ID NOS:45-46, respectively, the DNA comprising a base sequence coding for the L chain V region includes, for example, one represented by SEQ ID NO: 65, and the DNA comprising a base sequence coding for the H chain V region includes one represented by SEQ ID NO:57.

Further, the present invention is also directed to a DNA coding for said chimeric L or H chain. The DNA coding for said L chain includes, for example, one comprising the base sequence as shown in SEQ ID NO:65 and the DNA coding for said H chain includes one comprising the base sequence as shown in SEQ ID NO:57.

Still further, the present invention is also directed to a DNA comprising a base sequence coding for an L chain V region or H chain V region of said humanized antibody. The DNA comprising a base sequence coding for the L chain V region includes one comprising the base sequence as shown in any of SEQ ID NOS:66-74 and the DNA comprising a base sequence coding for the H chain V region includes one represented by SEQ ID NO:58.

The present invention also relates to a DNA for an L chain V region of a humanized antibody comprising a base sequence coding for the amino acid sequence as shown in

US 6,903,194 B1

7

any of SEQ ID NOs:47-55. Said DNA includes one comprising the base sequence as shown in any of SEQ ID NOs:66-74.

Still further, the present invention relates to a DNA for an H chain V region of a humanized antibody coding for the amino acid sequence as shown in SEQ ID NO:56. Said DNA includes one comprising the base sequence as shown in SEQ ID NO:58.

The present invention further relates to a recombinant vector comprising any of said DNAs.

The present invention still further relates to a transformant transformed with said recombinant vector.

Also, the present invention relates to a process for preparing a chimeric or humanized antibody against a human parathyroid hormone related protein comprising cultivating said transformant and collecting a chimeric or humanized antibody against a human parathyroid hormone related protein from the resulting culture.

Still further, the present invention also relates to a pharmaceutical composition, or hypercalcemia-suppressing or hypophosphatemia-improving agent comprising said antibody as an effective ingredient. The calcemia is caused by malignant tumor and the hypophosphatemia is often observed in patients suffering from hypercalcemia associated with malignant tumor. Thus, the antibody of the present invention can be used in the treatment of the malignant tumor or in the improvement of hypercalcemia or hypophosphatemia symptoms. The malignant tumor may include, but not limited to, at least one selected from the group consisting of pancreas, lung, pharynx, larynx, tongue, gingiva, esophagus, stomach, biliary duct, breast, kidney, urinary bladder, uterus and prostate cancers, and malignant lymphoma. The hypercalcemia-suppressing agent of the present invention can be applicable to any malignant tumor which may cause hypercalcemia.

The present invention will be described in detail hereinbelow.

1. Production of Mouse Monoclonal Antibodies Against Human PTHrP

Mouse monoclonal antibodies against PTHrP may be prepared by preparing hybridomas through cell fusion between myeloma cells and antibody-producing cells derived from animals immunized with the antigen and selecting clones producing antibodies specifically inhibiting the PTHrP activity from the resulting hybridomas.

(1) Preparation of Antigens

PTHrP used for the immunization of animals includes peptides having the whole or part of the amino acid sequence of PTHrP prepared by recombinant DNA technology or chemical synthesis, and PTHrP derived from supernatants of cancer cells causing hypercalcemia. For example, a peptide [PTHrP(1-34)] comprising the 1st to 34th amino acids of the known PTHrP (Kemp, B. E. et al., Science (1987) 238, 1568-1570) may be used as the antigen. The human PTHrP (1-34) has an amino acid sequence as shown in SEQ ID NO:75.

The resultant PTHrP is attached to a carrier protein such as thyroglobulin followed by addition of an adjuvant. Any adjuvant may be mixed, including Freund's complete and incomplete adjuvants.

(2) Immunization and Collection of Antibody Producing Cells

The above resultant antigen is administered to a mammal, such as mouse, rat, horse, monkey, rabbit, goat or sheep. Immunization may be carried out by any known methods, including intravenous, subcutaneous and intraperitoneal

8

injections. Intervals of injections for immunization are not particularly limited and may be a few days to a few weeks, preferably 4 to 21 days.

Two or three days after final immunization, antibody producing cells are collected. The antibody producing cells include spleen, lymph node and peripheral blood cells; generally, spleen cells are utilized. The single dose amount of antigen used for immunization is 100 µg per mouse.

(3) Determination of Antibody Titers

In order to determine the immune response levels of immunized animals and select hybridomas of interest from the cells subjected to cell fusion treatment, the antibody titer in the blood of the immunized animal or the antibody titer in the supernatant of the antibody producing cells is measured.

Methods for detecting the antibodies are known, including ELA (enzyme immunoassay), RIA (radio immunoassay), and ELISA (enzyme linked immunosorbent assay).

(4) Cell Fusion

Myeloma cells used to be fused with antibody producing cells include cell lines which are derived from various animals such as mouse, rat and human, and generally available for those skilled in the art. Suitable cell lines used are those having a drug resistance, incapable of surviving in a selective medium such as HAT medium in the unfused state, and capable of surviving therein only in the fused state. Generally used are 8-azaguanine resistant cell lines, which lack hypoxanthine-guanine-phosphoribosyltransferase and can not grow in a hypoxanthine-aminopterin-thymidine (HAT) medium.

Suitable myeloma cells to be used include various known cell lines, such as P3 (P3×63Ag8.653) (J. Immunol. (1979) 123:1548-1550); P3×63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81:1-7); NS-1 (Kohler, G. and Milstein, C., Eur. J. Immunol. (1976) 6:511-519); MPC-11 (Margulies, D. H. et al., Cell (1976) 8:405-415); SP2/0 (Shulman, M. et al., Nature (1978) 276:269-270); FO (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35:1-21); Si94 (Trowbridge, I. S., J. Exp. Med. (1978) 148:313-323); and R210 (Galfre, G. et al., Nature (1979) 277:131-133).

Antibody producing cells may be obtained from spleen cells, lymph node cells, or the like. That is, the spleen, lymph node or the like is extracted or removed from any of the aforementioned animals and the tissue is crushed. The resulting crushed materials are suspended in a medium or buffer, such as PBS, DMEM or RPMI 1640, filtered through stainless mesh or the like and centrifuged to prepare the desired antibody producing cells.

Then, said myeloma cells and antibody producing cells are subjected to cell fusion.

Cell fusion may be carried out by bringing the myeloma and antibody-producing cells into contact with each other at a ratio of 1:1 to 1:10 in a medium for the culture of animal cells, such as MEM, DMEM or RPMI-1640, in the presence of a fusion accelerator at 30 to 37° C. for 1 to 15 minutes. To accelerate the cell fusion, any fusion accelerator or virus can be used, such as polyethylene glycol with an average molecular weight of 1,000 to 6,000, polyvinyl alcohol or Sendai virus. The fusion of the antibody producing and myeloma cells may also be performed in a commercially available cell fusion apparatus utilizing an electric stimulation such as electroporation.

(5) Selection and Cloning of Hybridomas

Hybridomas of interest are selected from the cells after the cell fusion, for example, by a method utilizing selective growth of cells in selective media.

US 6,903,194 B1

9

That is, a cell suspension is diluted with a suitable medium and inoculated on a microtiter plate. A selective medium such as HAT medium is added to each well and incubated while properly replacing the selective medium with a fresh one.

Thus, the growing cells are collected as hybridomas.

These hybridomas are then screened by the limiting dilution, fluorescence-activated cell sorter or other method. Finally, hybridomas producing a monoclonal antibody are obtained.

(6) Collection of Monoclonal Antibodies

Methods for collecting monoclonal antibodies from the obtained hybridomas include conventional cell culture and ascites formation methods.

In the cell culture method, the hybridomas are cultivated in a medium for the culture of animal cells, such as RPMI-1640 medium containing 10 to 20% fetal bovine serum, MEM medium or serum-free medium, under conventional conditions (e.g., 37° C., 5% CO₂) for 2 to 14 days and the antibodies are collected from the supernatant.

In the formation of ascites, the hybridomas are inoculated intraperitoneally to the same species of mammal as the source of the myeloma cells so that the hybridomas grow abundantly. After 1 to 4 weeks, the ascites or sera are collected.

When the antibodies are necessary to be purified in these methods, known methods such as the ammonium sulfate precipitation, ion exchange chromatography and affinity chromatography may optionally be selected or combined.

2. Construction of Chimeric Antibodies

(1) Cloning of DNA Comprising Base Sequence Coding for V Region of Mouse Monoclonal Antibody Against Human PTHrP

(i) Preparation of mRNA

To clone DNA comprising a base sequence coding for V region of mouse monoclonal antibody against human PTHrP, the collected hybridomas are treated in a conventional manner, for example, guanidine-ultracentrifugation (Chirgwin, J. M. et al., *Biochemistry* (1979) 18, 5294-5299), or AGPC method (Chomczynski, P et al., *Analytical Biochemistry* (1987) 162, 156-159), to prepare the total RNA, from which mRNA is prepared by e.g. Oligo(dT)-cellulose span column attached to mRNA Purification Kit (Pharmacia)-Quick Prep mRNA Purification Kit (Pharmacia AB) can also be used to prepare mRNA without need of extraction of the total RNA.

(ii) Preparation and Amplification of cDNA

From the mRNA obtained in (i) above, each cDNA in the V regions of L and H chains is synthesized with the use of a reverse transcriptase. In the synthesis of cDNA, Oligo-dT primer or an other appropriate primer which hybridizes to L or H chain C region, for example, MHC2 primer having the base sequence as shown in SEQ ID NO:1, may be used.

In the cDNA synthesis, said mRNA and primer are mixed and the reaction is effected in the presence of a reverse transcriptase at e.g. 52° C. for 30 minutes.

Amplification of cDNA of both L and H chains can be performed by PCR (polymerase chain reaction) based on 5'-RACE method (Frohman; M. A. et al., *Proc. Natl. Acad. Sci. USA*, 85, 8998-9002, 1988; Belyavsky, A. et al., *Nucleic Acids Res.*, 17, 2919-2932, 1989) using 5'-Ampli FINDER RACE kit (CLONTECH Inc.). Thus, Ampli FINDER Anchor (SEQ ID NO:42) is linked to 5' end of the cDNA synthesized above and PCR is effected for DNAs comprising base sequences coding for L and H chain V regions. (Hereinafter, the DNA comprising a base sequence coding for L chain V region is sometimes referred to simply

10

as "DNA for L chain V region" or "DNA coding for L chain V region". This also applies to H chain V region, C region etc. similarly.)

The primer for amplifying DNA for L chain V region which may be used includes, for example, Anchor primer (SEQ ID NO: 2) and primers designed from conserved sequences in L chain constant region (C_L region) of mouse antibodies such as MLC primer having the base sequence as shown in SEQ ID NO:4. The primer for amplifying DNA for H chain V region which may be used includes, for example, Anchor primer (SEQ ID NO:2) and MHC-GI primer (SEQ ID NO:3) (S. T. Jones, et al., *Biotechnology*, 9, 88, 1991).

(iii) Purification of DNA and Determination of Base Sequence

The PCR products are subjected to agarose gel electrophoresis according to conventional procedures to excise DNA fragments of interest, which are then recovered, purified and ligated to a vector DNA.

Purification of DNA may be carried out using commercially available kits such as GENECLEAN II; BIO101. Vector DNA for carrying the DNA fragments which may be used herein is known, for example, pUC19 or Bluescript.

Said DNA and vector DNA are ligated using a known ligation kit (Takara Shuzo) to yield a recombinant vector. The resultant recombinant vector is introduced into e.g. *Escherichia coli* JM109 and ampicillin resistant colonies are selected; thus, a vector DNA is prepared in a known method: J. Sambrook, et al., "Molecular Cloning", Cold Spring Harbor Laboratory Press, 1989. After the vector DNA is digested with restriction enzyme(s), the base sequence of a desired DNA is determined by a known method such as dideoxy method: J. Sambrook, et al., "Molecular Cloning", Cold Spring Harbor Laboratory Press, 1989. In the present invention, an automated base sequence determining apparatus (DNA Sequencer 373A; ABI Inc.) may be used.

(iv) Complementarity Determining Region

H and L chain V regions form an antigen binding site and their whole structures have some similarity to each other. That is, four framework region (FR) portions are linked through three hypervariable regions, or complementarity determining region (CDR). The amino acid sequence in the FR is relatively well conserved while variability of the amino acid sequence in the CDR region is very high: Kabat, E. A. et al., "Sequence of Proteins of Immunological Interest" US Dept. Health and Human Services, 1983.

Many portions of the four FRs have β sheet structure and, as a result, three CDRs form a loop. The CDR may sometimes form a part of the β sheet structure. Therefore, three CDRs are sterically held at very near positions to each other by FRs, which form an antigen binding site together with the three CDRs in the paired regions.

In view of such facts, CDR regions can be found by comparison between the amino acid sequence in the variable region of a mouse monoclonal antibody against human PTHrP and the database of amino acid sequences for antibodies prepared by Kabat et al. ("Sequence of Proteins of Immunological Interest" US Dept. Health and Human Services, 1983) to investigate the homology therebetween.

(2) Construction of Expression Vector of Chimeric Antibody

Once DNA fragments coding for L and H chain V regions of mouse monoclonal antibody (hereinafter I or H chain of an antibody may sometimes be referred to as "mouse L chain" etc. for mouse antibodies and "human H chain" etc. for human antibodies) are cloned, the DNAs coding for mouse V regions and DNAs coding for human antibody constant regions are ligated and expressed to yield chimeric anti-human PTHrP antibodies.

US 6,903,194 B1

11

A standard method for preparing chimeric antibodies involves ligating a mouse leader sequence and V region sequence present in a cloned cDNA to a sequence coding for a human antibody C region already present in an expression vector of a mammalian cell. Alternatively, a mouse leader sequence and v region sequence present in a cloned cDNA are ligated to a sequence coding for a human antibody C region followed by ligation to a mammalian cell expression vector.

The polypeptide comprising human antibody C region can be any of H or L chain C regions of human antibodies, including, for example, Cy1, Cy2, Cy3 or Cy4 for human H chains or C α or C κ for L chains.

To prepare a chimeric antibody, two expression vectors are first constructed; that is, an expression vector containing DNAs coding for mouse L chain V region and human L chain C region under the control of an expression control region such as an enhancer/promoter system, and an expression vector containing DNAs coding for mouse H chain V region and human H chain C region under the control of an expression control region such as an enhancer/promoter system, are constructed. Then, host cells such as mammalian cells are cotransformed with these expression vectors and the transformed cells are cultivated in vitro or in vivo to produce a chimeric antibody: see, for example, WO91/16928.

Alternatively, the mouse leader sequence present in the cloned cDNA and DNAs coding for mouse L chain V region and human L chain C region as well as the mouse leader sequence and DNAs coding for mouse H chain V region and human H chain C region are introduced into a single expression vector (see, for example, WO94/11523) and said vector is used to transform a host cell; then, the transformed host is cultivated in vivo or in vitro to produce a desired chimeric antibody.

(i) Production of Chimeric Antibody H Chain

The vector for the expression of H chain of a chimeric antibody can be obtained by introducing cDNA comprising a base sequence coding for mouse H chain V region (hereinafter referred to also as "cDNA for H chain V region") into a suitable expression vector containing the genomic DNA comprising a base sequence coding for H chain C region of human antibody (hereinafter referred to also as "genomic DNA for H chain C region") or cDNA coding for said region (hereinafter referred to also as "cDNA for H chain C region"). The H chain C region includes, for example, Cy1, Cy2, Cy3 or Cy4 regions.

(i-a) Construction of Chimeric H Chain Expression Vector Containing Genomic DNA Coding for H Chain C Region

The expression vectors having the genomic DNA coding for H chain C region, in particular, those coding for Cy1 region, include, for example, HEF-PMh-g y1 (WO92/19759) and DHFR-A-E-RVh-PM1-f (WO92/19759).

When cDNA coding for mouse H chain V region is inserted into these expression vectors, an appropriate base sequence can be introduced into said cDNA through PCR method. For instance, PCR may be effected using a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 5'-end and Kozak consensus sequence immediately before the initiation codon thereof so as to improve the transcription efficiency, as well as a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 3'-end and a splice donor site for properly splicing the primary transcription products of the genomic DNA to give a mRNA, to introduce these appropriate base sequences into the expression vector.

12

After the thus constructed cDNA coding for mouse H chain V region is treated with a suitable restriction enzyme (s), it is inserted into said expression vector to construct a chimeric H chain expression vector containing the genome DNA coding for H chain C region (Cy1 region).

(i-b) Construction of Chimeric H Chain Expression Vector Containing cDNA Comprising Base Sequence Coding for H Chain

The expression vectors having the cDNA coding for H chain C region, such as Cy1 region, may be constructed in the following manner: mRNA is prepared from CHO cells into which an expression vector DHFR-AE-RVh-PM1-f (see WO92/19759) comprising DNA coding for H chain V region of a humanized PM1 antibody and genomic DNA of H chain C region Cy1 of a human antibody (N. Takahashi, et al., Cell, 29, 671-679 (1982)) and an expression vector RV1-PM1a (see WO92/19759) comprising genomic DNA coding for L chain V region of the humanized PM1 antibody and genomic DNA of L α chain C region of a human antibody have been introduced, and cDNA coding for the H chain V region of the humanized PM1 antibody and cDNA coding for the H chain C region (Cy1) of the human antibody are cloned by RT-PCR method and ligated to an animal cell expression vector which has been treated with a suitable restriction enzyme(s), to construct a desired expression vector.

When cDNA coding for mouse H chain V region is directly ligated to cDNA coding for H chain C region Cy1 of a human antibody, appropriate base sequences can be introduced into a fragment comprising cDNA coding for H chain V region through PCR method. For instance, PCR may be effected using a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 5'-end and Kozak consensus sequence immediately before the initiation codon thereof so as to improve the transcription efficiency, as well as a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 3'-end for directly ligating to the H chain C region Cy1, to introduce these appropriate base sequences into said cDNA.

The thus constructed cDNA coding for mouse H chain V region is treated with a suitable restriction enzyme(s), ligated to cDNA coding for said H chain C region Cy1, and inserted into an expression vector such as pCOS1 or pCHO1 to construct an expression vector containing the cDNA coding for a chimeric H chain.

(ii) Production of Chimeric Antibody L Chain

The vector for the expression of L chain of a chimeric antibody can be obtained by ligating a cDNA coding for mouse L chain V region and a genomic DNA or cDNA coding for L chain C region of a human antibody and introducing into a suitable expression vector. The L chain C region includes, for example, κ chain and λ chain.

(ii-a) Construction of Expression Vector Containing cDNA Coding for Chimeric L α chain

When an expression vector containing cDNA coding for mouse L chain V region is constructed, appropriate base sequences can be introduced into said expression vector through PCR method. For instance, PCR may be effected using a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 5'-end and Kozak consensus sequence for improving the transcription efficiency, as well as a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 3'-end, to introduce these appropriate base sequences into said cDNA.

The whole base sequence of cDNA coding for human L α chain C region may be synthesized by a DNA synthesizer.

US 6,903,194 B1

13

and constructed through PCR method. The human L λ chain C region is known to have at least 4 different isotypes and each isotype can be used to construct an expression vector. For example, based on a search for the homology with L λ chain C regions of cloned mouse monoclonal antibodies, an isotype Megg+Ke+Oz- of the fragment of human L λ chain C region (accession No. X57819) (P. Dariavach et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987) can be selected and used to construct an expression vector. To construct cDNA for the known human L λ chain C region such as Megg+Ke+Oz-, for example, the following four primers as shown in SEQ ID NOs:11-14 are designed: The primers MBC1HGP1 (SEQ ID NO:11) and MBC1HGP3 (SEQ ID NO:13) have sense DNA sequences and the primers MBC1HGP2 (SEQ ID NO:12) and MBC1HGP4 (SEQ ID NO:14) have antisense DNA sequences wherein each primer has a 20 to 23 bp complementary sequence at either end thereof.

MBC1HGPS (SEQ ID NO:15) and MBC1HGPR (SEQ ID NO: 16) are called external primers, have sequences homologous with MBC1HGP1 and MBC1HGP4, respectively, and have each a recognition sequence for a suitable restriction enzyme. Through PCR method, the four primers are assembled to synthesize full length cDNA and the external primers are added to amplify the cDNA.

The assembly through PCR method means that MBC1HGP1 and MBC1HGP2 or MBC1HGP3 and MBC1HGP4 are annealed through their complementary sequences to synthesize MBC1HGP1-MBC1HGP2 fragment and MBC1HGP3-MBC1HGP4 fragment and each fragment is again annealed through their complementary sequences to synthesize a cDNA coding for the full length human L λ chain C region.

The thus constructed cDNA coding for human L λ chain C region and the above constructed cDNA coding for mouse L chain V region can be ligated between suitable restriction enzyme sites and inserted into an expression vector such as pCOS1 or pCHO1 to construct an expression vector containing cDNA coding for a L λ chain of a chimeric antibody. (ii-b) Construction of Expression Vector Containing cDNA Coding for Chimeric L λ Chain

When an expression vector containing cDNA coding for mouse L chain V region is constructed, appropriate base sequences can be introduced into said cDNA through PCR method. For instance, PCR may be effected using a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 5'-end and Kozak consensus sequence for improving the transcription efficiency, and a PCR primer which is designed such that said cDNA has a recognition sequence for a suitable restriction enzyme at its 3'-end, to introduce these appropriate base sequences into said cDNA.

The DNA coding for human L λ chain C region to be ligated to the DNA coding for mouse L chain V region can be constructed from, for example, HEF-PM1k-gk containing the genomic DNA (see WO92/19759).

Recognition sequences for suitable restriction enzymes can be introduced, through PCR method, into 5'- and 3'-ends of DNA coding for L λ chain C region, and the DNA coding for mouse L chain V region as constructed above and the DNA excising for L λ chain C region can be ligated to each other and inserted into an expression vector such as pCOS1 or pCHO1 to construct an expression vector containing cDNA coding for L λ chain of a chimeric antibody.

3. Production of Humanized Antibodies

(1) Search for Homology With Human Antibodies

In order to make a humanized antibody in which CDR of a mouse monoclonal antibody is grafted to a human

14

antibody, it is desirable that there exists a high homology between FR of the mouse monoclonal antibody and FR of the human antibody. Accordingly, a comparison is made between V regions of H and L chains of mouse anti-human PTHrP monoclonal antibody and the V regions of all the known antibodies whose structures have been elucidated with the use of Protein Data Bank. Further, they are simultaneously compared with the human antibody subgroups (HSG: Human subgroup) classified by Kabat et al. based on the length of antibody FR, the homology of amino acids, and the like: Kabat, E. A. et al, US Dep. Health and Human Services, US Government Printing Offices, 1991.

Human H chain V regions may be classified into HSG I to III according to the HSG classification by Kabat et al. and mouse anti-human PTHrP monoclonal antibody H chain V regions have a homology of 82.7% with the consensus sequence of HSG III. On the other hand, human L λ chain V regions may be classified into HSG I to VI according to the HSG classification by Kabat et al. and mouse anti-human PTHrP monoclonal antibody L λ chain V regions do not have a high homology with the consensus sequences of human L λ chain V regions belonging to any subgroups.

When mouse anti-human PTHrP monoclonal antibody is to be humanized, therefore, it is desirable to use human H chain V region which belongs to HSG III and has the highest homology, or human H chain V region having a FR structure with a corresponding canonical structure (Chothia C. et al., J. Mol. Biol., 196, 901-917, 1987), as the human H chain V region, to construct a humanized antibody. Further, since there is no consensus sequence with a high homology in subgroups of human L λ chain V regions, it is desirable to use human antibody L λ chain V region with a highest homology registered in Protein Data Bank upon construction of a humanized antibody.

(2) Design of DNA Coding for Humanized Antibody V Region

The first step for designing DNA coding for a humanized antibody V region is to select a human antibody V region as a basis for the designing.

In the present invention, FR of a human antibody V region having a homology of higher than 80% with FR of a mouse antibody V region can be used in the humanized antibody. The FR of H chain V region as a fragment of a substantially identical FR may include FR derived from those belonging to the subgroup III, such as S31679: NBFR-PDB, Cuisinier A. M. et al., Eur. J. Immunol., 23, 110-118, 1993. Further, the FR of L chain V region as a fragment of a substantially identical FR may include, for example, FR1, FR2 and FR3 derived from human antibody HSU03868 (GEN-BANK, Deftos M. et al., Scand. J. Immunol., 39, 95-103, 1994) and FR4 derived from human antibody S25755 (NBFR-PDB).

The human antibody S31679 was cloned from cDNA library of human fetal livers while the human antibody HSU03868 was cloned as a novel gene for human L λ chain V region.

(3) Preparation of Polypeptides Comprising Humanized Antibody V Region

In the humanized antibody of the present invention, the C region and the framework (FR) regions of the V region of said antibody are originated from human and the complementarity determining regions (CDR) of the V region are originated from mouse (FIG. 1). A polypeptide comprising the V region of the humanized antibody according to the present invention can be made in the manner called CDR-grafting by PCR method so long as a DNA fragment of a human antibody would be available as a template. The "CDR-grafting" refers to a method wherein a DNA fragment

US 6,903,194 B1

15

coding for a mouse-derived CDR is made and replaced for the CDR of a human antibody as a template.

If a DNA fragment of a human antibody to be used as a template is not available, a base sequence registered in a database may be synthesized in a DNA synthesizer and a DNA for a V region of a humanized antibody can be made by the PCR method. Further, when only an amino acid sequence is registered in the database, the whole base sequence may be deduced from the amino acid sequence on the basis of knowledge on the codon usage in antibodies as reported by Kabat, E. A., et al. in US Dep. Health and Human Services, US Government Printing Office, 1991. This base sequence is synthesized in a DNA synthesizer and a DNA of a humanized antibody V region can be prepared by PCR method and introduced into a suitable host followed by expression thereof to produce the desired polypeptide.

Now, general procedures of CDR-grafting by PCR method are described below when a DNA fragment of a human antibody as a template is available.

(i) CDR-Grafting

Now suppose a DNA encoding V region comprises DNAs coding for FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 which are linked to each other in this order, as shown in FIG. 2.

First, mouse derived DNA fragments corresponding to respective CDRs are synthesized. CDRs 1 to 3 are synthesized on the basis of the base sequences of the previously cloned mouse H and L chain V regions. Grafting primers B and E are synthesized such that the primer B should have a sequence hybridizing to the mouse CDR1 and human antibody FR2 in the sense direction and the primer E should have a sequence hybridizing to the CDR1 and human antibody FR1 in the antisense direction (FIG. 2 (1)). Similarly, the grafting primers C and F and the primers D and G are synthesized. Further, suitable primers, called "external primers" and corresponding to A and H in FIG. 2 (1), which can hybridize to the regions upstream from FR1 and downstream from FR4, respectively, are also synthesized. Isolation and extraction of the grafting primers may be carried out in known procedures: Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989.

Then, first PCR is performed using the grafting primer E and external primer A, the grafting primers B and F, the grafting primers C and G as well as the grafting primer D and external primer H resulting in the formation of fragments A-E, B-F, C-G and D-H, respectively, (FIG. 2 (2)).

Since the upstream region of the grafting primer B and a part of the downstream region of the grafting primer E have been designed to overlap with each other (the same being true in the grafting primers C and F as well as D and G), these fragments may be annealed with respective complementary sequences by reacting under suitable temperature conditions and assembled to a DNA having a length from A to H by PCR. Once a DNA fragment coding for the V region is obtained, the external primers A and H may be added and second PCR may be performed to yield a DNA coding for the V region of a humanized antibody in which FRs 1 to 4 are human derived and CDRs 1 to 3 are mouse derived. Then, it may be introduced into a suitable host to express to yield the desired polypeptide (FIG. 2 (3)).

(ii) Construction of DNA and Expression Vector Coding for Humanized H Chain V Region

In the present invention, the whole base sequence of a DNA coding for H chain V region of a human antibody to be used as a template for a humanized antibody may be synthesized by a DNA synthesizer and constructed by PCR method although said DNA is not available from the natural source.

16

The H chain V region of a mouse anti-human PTHrP monoclonal antibody has a high homology with S31679 belonging to human subgroup III. In order to employ this human antibody as a template to construct a DNA coding for a humanized H chain V region, four primers as shown in SEQ ID NOs:23-26, for example, are used. The primers MBC1HGP1 (SEQ ID NO:23) and MBC1HGP3 (SEQ ID NO:24) have sense DNA sequences and MBC1HGP2 (SEQ ID NO:25) and MBC1HGP4 (SEQ ID NO:26) have antisense DNA sequences. They are designed to each have a 15 to 21 bp complementary sequence at either end thereof.

External primers MBC1HVS1 (SEQ ID NO:27) and MBC1HVR1 (SEQ ID NO:28) have a homologous sequence with MBC1HGP1 and MBC1HGP4, respectively, and each comprises a recognition sequence for a respective suitable restriction enzyme. The four primers are assembled by PCR method to synthesize a full length cDNA, and the external primers are added to amplify the DNA. The "assembling by PCR method" herein involves annealing MBC1HGP1 and MBC1HGP2 or MBC1HGP3 and MBC1HGP4 through their complementary sequences to synthesize a MBC1HGP1-MBC1HGP3 fragment and a MBC1HGP2-MBC1HGP4 fragment and further annealing the fragments through their complementary sequences to synthesize the full length DNA for a humanized H chain V region.

Human antibody H chain C region may be any human H chain C region such as, for example, human H chain C γ 1, C γ 2, C γ 3 or C γ 4.

The DNA for H chain V region of a humanized antibody constructed as above described may be ligated to DNA for any human antibody H chain C region, for example, human H chain C γ 1 region. As mentioned in the section "Production of H chain of chimeric antibody", the DNA for H chain V region may be treated with a suitable restriction enzyme and ligated to a DNA coding for a human H chain C region under an expression control region such as an enhancer/promoter system to make an expression vector containing DNAs for a humanized H chain V region and a human H chain C region.

(iii) Construction of DNA and Expression Vector Coding for Humanized L Chain V Region

In the present invention, the whole base sequence of DNA coding for L chain V region of a human antibody to be used as a template may be synthesized by a DNA synthesizer and constructed by PCR method although the DNA for L chain V region is not available as in the case of the DNA coding for H chain V region.

In order to construct a DNA for a humanized L chain V region using as a template a human antibody SU03868 having a highest homology with the L chain V region of a mouse anti-human PTHrP monoclonal antibody, four primers as shown in SEQ ID NOs:29-32, for example, are used. The primers MBC1LGP1 (SEQ ID NO:29) and MBC1LGP3 (SEQ ID NO:30) have sense DNA sequences and MBC1LGP2 (SEQ ID NO:31) and MBC1LGP4 (SEQ ID NO:32) have antisense DNA sequences. They are designed to each have a 15 to 21 bp complementary sequence at either end thereof.

External primers MBC1LVS1 (SEQ ID NO:33) and MBC1LVR1 (SEQ ID NO:34) have a homologous sequence with MBC1LGP1 and MBC1LGP4, respectively, and each comprises a recognition sequence for a respective suitable restriction enzyme. The four primers are assembled by PCR method to synthesize a full length DNA, and the external primers are added to amplify the DNA. The "assembling by PCR method" herein involves annealing MBC1LGP1 and

US 6,903,194 B1

17

MBC1LGP3 or MBC1LGP2 and MBC1LGP4 through their complementary sequences to synthesize a MBC1LGP1-MBC1LGP3 fragment and a MBC1LGP2-MBC1LGP4 fragment and further annealing the fragments through their complementary sequences to synthesize a full length DNA coding for a humanized H chain V region.

Human antibody L chain C region may be any human L chain C region such as, for example, human L chain C γ or C κ .

The DNA for L chain V region of a humanized antibody constructed as above described may be ligated to DNA for any human antibody L chain C region, for example, human L chain CL region. The DNA for L chain V region may be treated with a suitable restriction enzyme and ligated to a DNA coding for a human L chain C region under an expression control region such as an enhancer/promoter system to make an expression vector containing DNAs coding for a humanized L chain V region and a human L γ chain C region.

Even if a polypeptide comprising a V region of a humanized antibody could be produced as above described, it is not necessarily clear whether or not said polypeptide would have an activity as an antibody, such as binding or neutralizing activity against its antigen. Particularly in the case of L chain, since the L chain V region of a mouse anti-human P1HrP monoclonal antibody is derived from a very rare V λ -X gene, it should be necessary to investigate the presence or absence of the activity by combining it with a humanized H chain and expressing in an animal cell such as COS-7.

As a method for elucidating which FR in a humanized antibody V region may contribute to the binding and neutralizing activity of the humanized antibody, construction of a hybrid V region (Ohtomo, T. et al., Molecular Immunology, 32, 407-416, 1995) and confirmation may be effective. In order to elucidate which amino acid in the L chain V region of the humanized antibody according to the present invention should be mutated to provide one having the activity, a DNA in which a fragment of an FR region of a humanized antibody is recombined with a fragment of a mouse derived FR region is constructed and each region is assessed for humanization.

As shown in FIG. 3, an antibody having a polypeptide comprising a recombinant V region in which FR1 and FR2

18

are derived from a human antibody and FR3 and FR4 are derived from a mouse antibody (such an antibody having a recombinant fragment being referred to as a "hybrid antibody"), a hybrid antibody in which only FR1 is human derived, and a hybrid antibody in which only FR2 is human derived, are made. Each of DNAs coding for these hybrid antibodies is introduced into an expression vector and the humanized antibodies are temporarily expressed to investigate the presence of antibody activities.

Using this method, the present inventor has investigated polypeptides comprising L chain V regions for antigen binding and neutralizing activities and finally found that certain amino acids to be replaced exist in FR2 and FR3.

Having found that amino acids contributing to the activity exist in FR2 and FR3 regions, the present inventor has elucidated that the 36th, 45th and 49th amino acids in FR2 region and the 87th amino acid in FR3 region (the numbering of amino acids of antibodies having been determined by Kabat, E. A. et al., US Dep. Health and Human Services, US Government Printing Offices, 1991) contribute to the activity.

Thus, a polypeptide comprising a V region in which such amino acid(s) is/are mutated (e.g., replaced) is made in the present invention.

First, a polypeptide comprising a V region having an amino acid sequence as a base into which a mutation of amino acid(s) is to be introduced is prepared by the aforementioned CDR-grafting. This base polypeptide comprises the amino acid sequence as shown in SEQ ID NO:47 and is referred to as "version a" (a in Table 1).

Then, from the version a as a base, various variant fragments in which one or some amino acids of FR are mutated are made.

The introduction of mutation may be carried out by designing an oligonucleotide primer (mutagenic primer) coding for an amino acid to be introduced as a desired mutation and performing PCR using said primer.

Thus, polypeptides comprising V regions (versions b to t) in which a specific amino acid(s) in FR2 and FR3 is/are mutated are made (b to t in Table 1).

Table 1:

TABLE 1

	FR1			CDR1		
		1	2	3		
		123456789012345678901234567890		12345		
MEC H. PEP		EVQLVESGGDLVPGGSLKLSCAASGFTFS		SYGMS		
		* * * * *				
S31679		QVQLVESGGGVVQPGSLRLSCAASGFTFS		SYAMH		
hMBCI-H. pep		-----		SYGMS		
	FR2		CDR2			
		4	5	6		
		67890123456789	012A3456789012345			
MEC H. PEP		WIRQTPDKRLWVA	TISGGSTTYTPDSVKG			
		* * * *				
S31679		WVRQAPGKGLWVA	VISYDGSNKYYADSVKG			
hMBCI-H. pep		-----	TISGGSTTYTPDSVKG			
	FR3			CDR3	FR4	
		7	8	9	10	11
		67890123456789012ABC345678901234		567890A12	567890123	
MEC H. PEP		RFTISRDNKNTLYLQMSLSKEDTAMFYCAR		QTTMTYPAY	WGQGLVTVSS	
		* * * *				
S31679		RFTISRDNKNTLYLQMSLSRAEDTAVTYCAR		ESRGDY	WGQGLVTVSS	
hMBCI-H. pep		-----		QTTMTYPAY		

US 6,903,194 B1

19

20

TABLE 1-continued

	PR1	1	2	CDR1	3	PR2	4	CDR2	5
		12345678901234567890123			45678901234		567890123456789		0123456789
HBC L. PEP		QLVLTQSSS--ASFSLGASAKLTC			TLSSQHSHTYITE		WYQQQPLKPPKYVMD		LKQDGSHTGTD
		o * *					* * * *		
HSU03868		QLVLTQSPF--ASASLGASVKLTC			TLSSGHSYATA		WHQQQPEKGPPLYLMK		LMSDGSHSKED
hMBC1-L. pep					TLSSQHSHTYITE				LKQDGSHTGTD
a		-----			-----		-----P-----D		-----
b		-----			-----		-----		-----
c		-----			-----		-----P-----D		-----
d		-----			-----		-----		-----
e		-----			-----		-----P-----D		-----
f		-----			-----		-----		-----
g		-----			-----		-Y-----		-----
h		-----			-----		-Y-----K-----		-----
i		-----			-----		-Y-----K-----D		-----
j		-----			-----		-Y-----K-----V-----		-----
k		-----			-----		-Y-----K-----V-----D		-----
l		-----			-----		-Y-----V-----		-----
m		-----			-----		-Y-----V-----D		-----
n		-----			-----		-Y-----K-----		-----
o		-----			-----		-Y-----K-----D		-----
p		-----			-----		-Y-----K-----D		-----
q		-----			-----		-Y-----K-----D		-----
r		-----			-----		-Y-----K-----V-----D		-----
s		-----			-----		-Y-----K-----V-----D		-----
t		-----			-----		-Y-----V-----D		-----
	PR3	6	7	8	CDR3	9	PR4	10	11
		78901234567890123456789012345678				90123456789012345678901234567890			
HBC L. PEP		GIPDRFGSSSGADRYLSISNIQPEDEAMNYIC				GVGDTIKEQFVYV			FGGGTKVTVLGQP
		* * * *							
HSU03868		GIPDRFGSSSGAERYLYTISLQSEDEADTYIC			QTWGTGI				
hMBC1-L. pep					GVGDTIKEQFVYV				
a		-----			-----L-----				
b		-----			-----L-----				
c		-----P-----			-----L-----				
d		-----I-----			-----L-----				
e		-----L-----			-----L-----				
f		-----P-----I-----			-----L-----				
g		-----			-----L-----				
h		-----I-----			-----L-----				
i		-----			-----L-----				
j		-----			-----L-----				
k		-----			-----L-----				
l		-----			-----L-----				
m		-----			-----L-----				
n		-----			-----L-----				
o		-----I-----			-----L-----				
p		-----I-----			-----L-----				
q		-----I-----			-----L-----				
r		-----I-----			-----L-----				
s		-----I-----			-----L-----				
t		-----I-----			-----L-----				

The DNA coding for each version of L chain V region of a humanized antibody as constructed above may be ligated to a DNA of any L chain C region of a human antibody, such as human L chain C α region. Thus, it is treated with a suitable restriction enzyme and ligated to a DNA coding for a human L α chain C region under the control of an expression control region such as an enhancer/promoter system to construct an expression vector comprising a DNA coding for each version of the humanized L chain V region and a DNA coding for the humanized L α chain C region.

The DNA coding for H chain V region of a humanized antibody and a human H chain C region as previously constructed and the DNA coding for a humanized L chain V region and human L chain C region may also be introduced into a single expression vector such as that disclosed in WO/94/11523, said vector may be used to transform a host cell, and the transformed host may be cultivated in vivo or in vitro to produce a desired humanized antibody.

4. Production of Chimeric Antibody and Humanized Antibody

To produce a chimeric or humanized antibody, two expression vectors as above mentioned should be prepared. Thus, with respect to a chimeric antibody, an expression vector comprising a DNA coding for a mouse H chain V region and a human H chain C region under the control of an expression control region such as an enhancer/promoter system, and an expression vector comprising a DNA coding for a mouse L chain V region and a human L chain C region under the control of an expression control region such as an enhancer/promoter system are constructed. With respect to a humanized antibody, an expression vector comprising a DNA coding for a humanized H chain V region and a human H chain C region under the control of an expression control region such as an enhancer/promoter system, and an expression vector comprising a DNA coding for a humanized L chain V region and a human L chain C region under the

US 6,903,194 B1

21

control of an expression control region such as an enhancer/promoter system are constructed.

Then, a host cell such as a mammalian cell is cotransformed with these expression vectors and the resulting transformed cell is cultivated in vitro or in vivo to produce the chimeric or humanized antibody (see, for example, WO91/16928).

Alternatively, a DNA coding for H chain V and C regions and a DNA coding for L chain V and C regions may be ligated to a single vector and transformed into a suitable host cell to produce an antibody. Thus, in the expression of a chimeric antibody, a DNA coding for a mouse leader sequence present in the cloned cDNA, a mouse H chain V region and a human H chain C region as well as a DNA coding for a mouse leader sequence, a mouse L chain V region and a human L chain C region, are introduced into a single expression vector such as one disclosed in e.g. WO94/11523. In the expression of a humanized antibody, a DNA coding for a humanized H chain V region and a human H chain C region and a DNA coding for a humanized L chain V region and a human L chain C region are introduced into a single expression vector such as one disclosed in e.g. WO94/11523. Such a vector is used to transform a host cell and the transformed host is cultivated in vivo or in vitro to produce a chimeric or humanized antibody of interest.

The chimeric or humanized antibody of interest which is thus produced by cultivating the transformant transformed with a DNA coding for said chimeric or humanized antibody may be isolated from the interior or exterior of the cell and purified to uniformity.

The isolation and purification of the chimeric or humanized antibody of interest according to the present invention may be carried out by using a protein A agarose column, but may also be performed by any methods used in isolation and purification of conventional proteins and thus is not limited. For instance, various chromatography, ultrafiltration, salting out and dialysis may optionally be selected or combined to isolate and purify the chimeric or humanized antibody.

Any expression system may be used to produce the chimeric or humanized antibody against human PTHrP according to the present invention. For example, eukaryotic cells include animal cells such as established mammalian cell lines, mold and fungal cells, and yeast cells; prokaryotic cells include bacterial cells such as *Escherichia coli* cells. Preferably, the chimeric or humanized antibody of the present invention is expressed in a mammalian cell such as COS or CHO cell.

Any conventional promoters useful for the expression in mammalian cells may be used. For example, human cytomegalovirus immediate early (HCMV) promoter is preferably used. Examples of expression vectors comprising HCMV promoter include HCMV-VH-HC γ 1 and HCMV-VL-HCK derived from pSV2neo (WO92/19759).

In addition, promoters for gene expression in mammalian cells which can be used in the present invention may include virus promoters, such as those of retrovirus, polyoma virus, adenovirus and simian virus (SV) 40, and mammalian cell derived promoters, such as those of human polypeptide chain elongation factor-1 α (IEF-1 α). For example, SV40 promoter may be readily used according to Mulligan et al. method (Nature, 277, 108, 1979); Mizushima, S. et al. method (Nucleic Acids Research, 18, 5322, 1990) may be easily used with IEF-1 α promoter.

Origin of replication usable herein includes those derived from SV40, polyoma virus, adenovirus or bovine papilloma virus (BPV). Further, the expression vector may comprise a gene for phosphotransferase APT(3') II or I (neo), thymidine

22

kinase (TK), *E. coli* xanthine-guanine phosphoribosyltransferase (Ecogpt) or dihydrodipicolinate reductase (DHFR) as a selective marker for increasing the gene copy number in a host cell system.

5 Evaluation of Antigen Binding and Neutralizing Activity of Chimeric and Humanized Antibodies

(1) Determination of Antibody Concentration

The concentration of the resulting purified antibody can be determined by ELISA.

10 ELISA plates for determining the antibody concentration are prepared in the following manner: 100 μ l of a goat anti-human IgG antibody prepared at a concentration of e.g. 1 μ g/ml is immobilized in each well of a 96 well plate for ELISA (for example, Maxisorp, NUNC). After blocking with 200 μ l of a diluting buffer (for example, 50 mM Tris-HCl, 1 mM MgCl $_2$, 0.1 M NaCl, 0.05% Tween20, 0.02% NaN $_3$, 1% bovine serum albumin (BSA), pH 7.2), a stepwise diluted supernatant of COS-7 or CHO cells in which the chimeric, hybrid or humanized antibody has been expressed, or purified chimeric, hybrid or humanized antibody is added to each well, 100 μ l of an alkaline phosphatase-conjugated goat anti-human IgG antibody is added, and 1 mg/ml of a substrate solution (Sigma 104, p-nitrophenylphosphoric acid, SIGMA) is then added, after which the absorbance at 405 nm is measured by a microplate reader (Bio Rad). Hu IgG1 α Purified (The Binding Site) may be used as a standard for the determination of concentrations.

(2) Determination of Antigen Binding Ability

15 ELISA plates for determining the antigen binding ability are prepared in the following manner: 100 μ l of human PTHrP (1-34) prepared at a concentration of 100 μ g/ml is immobilized to each well of a 96 well plate for ELISA. After blocking with 200 μ l of a diluting buffer, a stepwise diluted supernatant of COS-7 or CHO cells in which the chimeric, hybrid or humanized antibody has been expressed, or purified chimeric, hybrid or humanized antibody is added to each well, 100 μ l of an alkaline phosphatase-conjugated goat anti-human

(3) Determination of Neutralizing Activity

20 Determination of the neutralizing activity of the mouse, chimeric and humanized antibodies can be carried out by, e.g., using rat osteosarcoma cell line ROS17/2.8-5 cell (Sato, K. et al., Acta Endocrinologica, 116, 113-120, 1987). Thus, ROS17/2.8-5 cells are stimulated by 4 mM hydrocortisone to induce PTH/PTHrP receptor. The degradative enzyme for cAMP is inhibited with 1 mM of isobutyl-1-methyl xanthine (IBMX, SIGMA). The mouse, chimeric or humanized antibody to be determined for neutralizing activity is mixed with an equal amount of PTHrP (1-34) and the resulting mixture of each antibody and PTHrP (1-34) is added to each well. The neutralizing ability of the mouse, chimeric or humanized antibody can be estimated by measuring the amount of cAMP produced by rat osteosarcoma cell lines ROS17/2.8-5 cells due to stimulation with PTHrP.

25 (4) Kinetic Analysis of Interactions Between PTHrP and Anti-PTHrP Antibody

In the present invention, the kinetics in the interactions between PTHrP and anti-PTHrP may be analyzed by a variety of means and procedures. Specifically, dissociation constants, dissociation rate constants and association rate constants may be measured by Scatchard analysis and a surface plasmon resonance sensor called BIACORE (developed and commercialized by Pharmacia Biotech). Analysis by a surface plasmon resonance sensor called BIACORE will be described hereinbelow as one example.

The basic structure of BIACORE comprises an optical source, a prism, a detector and a micro-passage. In practice,

US 6,903,194 B1

23

a ligand is immobilized on a cassette-type sensor tip and an analyte is injected thereto. When there is any affinity between them, the binding amount is optically detected.

The detecting principle is a phenomenon called surface plasmon resonance. Thus, of incident light injected to the interface between a glass and a metal film so that total reflection should occur, the incident light at a certain angle is used to excite surface plasmon and damped. The angle varies depending upon the change in concentration of a solvent in contact with the metal film (sensor). BIACORE detects this change.

In BIACORE, this change is called a resonance signal (SPR signal) and a change of 0.1 degree is 1000 RU (resonance units). 1000 RU corresponds to a change in the binding of about 1 ng of a protein onto a thin gold sensor of 1 mm² in surface area. For a protein, a change of about 50 RU (50 pg) can be fully detected.

The detected signals are converted into a binding curve called a sensogram by a computer attached to BIACORE, which is drawn on a computer display in real time: Natsume, T., et al. (1995) *Experimental Medicine*, 13, 563-569; Karlsson, R., et al. (1991) *J. Immunol. Methods* 145, 229-240.

Kinetics parameters, i.e., dissociation constant (KD), dissociation rate constant (Kdiss) and association rate constant (Kass), of the anti-PTHrP antibodies of the present invention may be measured by the above mentioned BIACORE.

The anti-PTHrP antibodies of the present invention preferably have as small a dissociation constant (KD value) as possible in view of neutralizing activity. Preferably, the anti-PTHrP antibodies of the present invention have a KD value 1.86x10⁻⁷ or less, more preferably 1.86x10⁻⁸ or less, most preferably 3.58x10⁻¹⁰ or less.

The KD values are determined from two parameters, dissociation rate constants (Kdiss) and association rate constants (Kass) (KD=Kdiss/Kass). Apparently, therefore, the KD values are smaller when the Kdiss values are smaller and the Kass values are larger.

Specifically, the Kdiss values of the anti-PTHrP antibodies according to the present invention may be 1.22x10⁻¹ [1/Sec] or less. Preferably, the Kdiss values are 1.22x10⁻² or less, more preferably 3.16x10⁻⁴ or less, most preferably 2.32x10⁻⁴ [1/Sec] or less.

On the other hand, the Kass values may be 6.55x10⁴ [1/M.Sec] or more. Preferably, the Kass values are 6.55x10⁵ or more, more preferably 0.883x10⁶ or more, most preferably 1.03x10⁶ [1/M.Sec] or more.

Further, also preferred are anti-PTHrP antibodies having a Kdiss value of 1.22x10⁻¹ [1/Sec] and a Kass value of 6.55x10⁴ [1/M.Sec] or more.

More specifically, the anti-PTHrP antibodies of the present invention have a KD value in the range of 1.02x10⁻¹¹ to 1.86x10⁻⁷ [M], preferably 1.02x10⁻¹⁰ to 1.86x10⁻⁸ [M], more preferably 1.34x10⁻¹⁰ to 3.58x10⁻¹⁰ [M], most preferably 2.25x10⁻¹⁰ to 3.58x10⁻¹⁰ [M].

The Kdiss values are in the range of 7.38x10⁻⁵ to 1.22x10⁻¹ [1/Sec], preferably 7.38x10⁻⁵ to 1.22x10⁻² [1/Sec], more preferably 1.66x10⁻⁴ to 3.16x10⁻⁴ [1/Sec], most preferably 1.66x10⁻⁴ to 2.32x10⁻⁴ [1/Sec].

The Kass values are in the range of 6.55x10⁴ to 1.24x10⁷ [1/M.Sec], preferably 6.55x10⁵ to 1.24x10⁶ [1/M.Sec], more preferably 7.23x10⁵ to 1.03x10⁶ [1/M.Sec], most preferably 0.883x10⁶ to 1.03x10⁶ [1/M.Sec].

These KD, Kdiss and Kass values may be obtained by Scatchard analysis or a surface plasmon resonance sensor such as BIACORE, preferably by BIACORE.

6. Pharmaceutical Composition and Hypercalcemia-Suppressing Agent Comprising Anti-PTHrP or Humanized Antibody as Effective Ingredient.

24

The therapeutic effect of the humanized antibody on PTHrP may be confirmed by administering the antibody against PTHrP or the humanized antibody to an animal exhibiting hypercalcemia and measuring an index for hypercalcemia. In animals exhibiting hypercalcemia and patients suffering from hypercalcemia, hypophosphatemia is often observed; the antibodies of the present invention may also be used to improve the hypophosphatemia.

The antibody used in the present invention is an anti-PTHrP antibody including human, chimeric and primatized antibodies or a humanized antibody against PTHrP having the dissociation constant, dissociation rate constant and association rate constant. The antibody will neutralize the activity of PTHrP by binding to PTHrP and preferably includes, in particular, humanized #23-57-137-1 antibody. The method for producing the humanized #23-57-137-1 antibody will be described in Examples 1 to 3.

The antibody used in the present invention can be purified to a high purity by any combination of conventional purification means such as salting out, gel filtration using HPLC etc., and affinity chromatography using a protein A column etc. Recognition of PTHrP by the thus purified antibody with a high accuracy may be confirmed by any conventional immunological means such as radioimmunoassay (RIA), enzyme immunoassay (EIA, ELISA) or immunofluorescence analysis.

The animal exhibiting hypercalcemia which may be used includes a model animal prepared by transplanting PTHrP-producing tumor cells to an experimental animal with reduced or deleted immunological function. The tumor cells transplanted are preferably human derived ones including, for example, human pancrea cancer PAN-7. The animal with reduced or deleted immunological function to which the tumor cells are transplanted includes nude mouse and SCID mouse.

Suppression of hypercalcemia may be evaluated by observing the concentration of calcium in the blood, the reduction of body weight or the reduction of extent of movement with the lapse of time and determining the degree of improvement.

The pharmaceutical composition and hypercalcemia suppressing agent comprising the antibody or humanized antibody against PTHrP as an effective ingredient according to the present invention may be parenterally administered systemically or topically. For example, intravenous injection including drip, intramuscular injection, intraperitoneal injection or subcutaneous injection may be selected. The method of administration may be properly selected depending on the age of a patient and the conditions of disease. An effective single dose may be selected from the range of 0.01 to 1,000 mg per kg of body weight. Alternatively, the dose to a patient may be 5 to 10,000 mg/body, preferably 50 to 1,000 mg/body.

The pharmaceutical composition and hypercalcemia suppressing agent comprising the antibody or humanized antibody against PTHrP as an effective ingredient according to the present invention may further comprise a pharmaceutically acceptable carrier and/or additive(s) depending upon the administration route. Examples of such carrier and additive may include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinyl pyrrolidone, carboxyvinyl polymer, sodium carboxymethyl cellulose, poly(sodium acrylate), sodium arginate, water soluble dextran, sodium carboxymethyl starch, pectin, methyl cellulose, ethyl cellulose, xanthane gum, gum arabic, casein, gelatin, agar, diglycerin, glycerin, propylene glycol, polyethylene glycol, vaseline, paraffin, stearyl alcohol,

US 6,903,194 B1

25

stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, and surfactants acceptable as pharmaceutical additives. The additives used may be properly selected from the above either alone or in combination, but not limited thereto.

The antibody of the present invention may be used widely in hypercalcemia associated with various cancers (malignant tumors). These cancers are not particularly limited and include not only a single cancer but also a combination of a plurality of cancers. The cancers may include for example pancreas, lung, pharynx, larynx, tongue, gingiva, esophagus, stomach, biliary duct, breast, kidney, urinary bladder, uterus and prostate cancers, and malignant lymphoma.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic illustration of the antibody according to the present invention;

FIG. 2 is a schematic illustration of the CDR-grafting;

FIG. 3 is an illustration of the determination of the FRs and the CDRs of the V region;

FIG. 4 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 5 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 6 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 7 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 8 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 9 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 10 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 11 is a graph showing the measurement result of the antigen-binding activity of the antibodies;

FIG. 12 is a graph showing the neutralizing activity of the humanized antibodies;

FIG. 13 is a graph showing the neutralizing activity of the humanized antibodies;

FIG. 14 is a graph showing the neutralizing activity of the humanized antibodies;

FIG. 15 are graphs illustrating the efficacy of the antibodies of the present invention against a hypercalcemic model animal;

FIG. 16 are graphs illustrating the efficacy of the antibodies of the present invention on a hypercalcemic model animal;

FIG. 17 are graphs illustrating the efficacy of the antibodies of the present invention on a hypercalcemic model animal;

FIG. 18 are graphs illustrating the efficacy of the antibodies of the present invention on a hypercalcemic model animal;

FIG. 19 is a sensorgram illustrating the immobilization of PTHrP onto sensor tip;

FIG. 20 is a graph showing the results of the kinetic analysis of the antibody according to the invention;

FIG. 21 is a graph showing the results of the kinetic analysis of the antibody according to the invention;

FIG. 22 is a graph showing the results of the kinetic analysis of the antibody according to the invention;

FIG. 23 is a graph showing the results of the kinetic analysis of the antibody according to the invention;

26

FIG. 24 is a graph showing the results of the kinetic analysis of the antibody according to the invention;

FIG. 25 is a graph showing the test results of the effect of the humanized antibody according to the invention on fractional excretion of phosphate;

FIG. 26 is a graph showing the test result of the effect of the humanized antibody according to the invention on phosphorus concentration in plasma;

FIG. 27 is a photograph showing the apparent clinical symptoms of hypercalcemia model mice developed after administration of the anti-PTHrP antibody according to the invention (morphology of living animal);

FIG. 28 is a photograph showing the apparent clinical symptoms of hypercalcemia model mice developed after administration of the anti-PTHrP antibody according to the invention (morphology of living animal);

FIG. 29 is a graph showing the change in spontaneous activity of a hypercalcemia model animal over time after administering the anti-PTHrP antibody according to the invention in comparison with that of a control model animal administered with physiological saline;

FIG. 30 is a graph showing the change in body temperature of a hypercalcemia model animal over time after administering the anti-PTHrP antibody according to the invention in comparison with that of a control model animal administered with physiological saline; and

FIG. 31 is a graph showing the change in blood pH of a hypercalcemia model animal over time after administering the anti-PTHrP antibody according to the invention in comparison with that of a control model animal administered with physiological saline.

EXAMPLE

Hereinbelow, the present invention will be described in more detail with reference to the following Examples, which should not be construed as limiting the scope of the invention.

Reference Example 1

Preparation of Anti-PTHrP (1-34) Mouse Monoclonal Antibody Producing Hybridoma

Hybridomas capable of producing a monoclonal antibody against human PTHrP (1-34), #23-57-154 and #23-57-137-1, were prepared in accordance with the method reported by Kanji Sato et al. (Sato, K. et al., J. Bone Miner. Res. 8, 849-860, 1993).

The immunogen used was PTHrP (1-34) (Peninsula), to which a carrier protein, thyroglobulin, was conjugated using carbodiimide (Dojinn). The thyroglobulin-conjugated PTHrP (1-34) was dialyzed to obtain a solution having a protein concentration of 2 μ g/ml. The resultant solution was mixed with Freund's adjuvant (Difco) in a mixing ratio of 1:1 to obtain an emulsion. This emulsion was injected to each of 16 female BALB/C mice dorsal-subcutaneously or intraperitoneally in a dose amount of 100 μ g/mouse to immunize the mice. The injection was conducted 11 times. With respect to the adjuvant, Freund's complete adjuvant was used in the injection for the first immunization, and Freund's incomplete adjuvant was used in the injection for subsequent immunizations.

Each of the mice immunized was determined for its antibody titers in the sera in the following manner:

Each of the mice was blood-drawn from its tail vein and the blood was then subjected to centrifugation to obtain a

US 6,903,194 B1

27

serum. The serum was diluted with a RIA buffer, mixed with ¹²⁵I-labeled PTHrP (1-34), and subjected to determination of its binding activity. The mice which have been confirmed to have a satisfactorily high antibody titer were injected with PTHrP (1-34) without the carrier protein intraperitoneally in a dose amount of 50 µg/mouse for the final immunization.

Three days after the final immunization, the mice were sacrificed and excised their spleens. Thereafter, spleen cells were subjected to cell fusion with mouse myeloma cell line P3x63Ag8U.1 in accordance with a conventional known method using 50% polyethylene glycol 4000. The fused cells thus prepared were inoculated into each well of 85 of 96-well plates in an amount of 2x10⁵/well. The screening of hybridomas of interest was conducted using a HAT medium as follows.

The screening of the hybridomas was conducted by determining for the presence of PTHrP-recognition antibodies in the culture supernatant with respect to the wells in which cell growth had been observed in the HAT medium by a solid phase RIA method. The hybridomas were collected from the wells in which the binding ability to the PTHrP-recognition antibody was confirmed. The hybridomas thus obtained was suspended into a RPMI-1640 medium containing 15% FCS and supplemented with OPI-supplement (Sigma), followed by unification of the hybridomas by a limiting dilution method, thereby obtaining two types of hybridoma clones, #23-57-154 and #23-57-137-1 both exhibiting a strong binding ability to PTHrP (1-34).

Hybridoma clone #23-57-137-1, which was designated "mouse—mouse hybridoma #23-57-137-1", has been deposited under the terms of the Budapest Treaty on Aug. 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaragi-ken, Japan) under the accession No. FERM BP-5631.

EXAMPLE 1

Cloning of DNA Coding for V Region of Mouse Monoclonal Antibody Against Human PTHrP (1-34)

Cloning of the DNA coding for the V region of the mouse monoclonal antibody against human PTHrP (1-34) obtained above, #23-57-137-1, was conducted in the following manner.

(1) Preparation of mRNA

mRNA was prepared from hybridoma #23-57-137-1 using Quick Prep mRNA Purification Kit (Pharmacia Biotech) as follows.

The cells of hybridoma #23-57-137-1 obtained above were fully homogenized with an extraction buffer, and mRNA was extracted therefrom using an oligo(dT)-Cellulose Spin Column in accordance with the procedure by the manufacturer of the kit. The extraction solution was subjected to ethanol precipitation to obtain the mRNA as precipitates. The mRNA precipitates were dissolved in an elution buffer.

(2) Preparation and Amplification of cDNA of the Gene Coding for Mouse H Chain V Region

(i) Cloning of cDNA for H Chain V Region of #23-57-137-1 Antibody

A DNA coding for the H chain V region of the mouse monoclonal antibody against human PTHrP was cloned by a 5'RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). This method was

28

conducted using 5'-Ampli FINDER RACE Kit (CLONETECH) in accordance with the procedure by the manufacturer. In this method, the primer used for synthesis of cDNA was MHC2 primer (SEQ ID NO: 1) which is hybridizable with mouse H chain C region. About 2 µg of the above-obtained mRNA, which was a template for cDNA synthesis, was mixed with 10 pmoles of MHC2 primer. The resultant mixture was reacted with a reverse transcriptase at 52° C. for 30 min to prepare a cDNA which was complementary to the mRNA.

The resultant was mixed with 6N NaOH to hydrolyze the mRNA therein (at 65° C. for 30 min.) and then subjected to ethanol precipitation to isolate the cDNA as precipitates. The cDNA thus isolated was ligated to Ampli FINDER Anchor (SEQ ID NO: 42) on its 5'-end by reacting with T4 RNA ligase at 37° C. for 6 hours and additionally at room temperature for 16 hours. As the primers for amplification of the cDNA by a PCR method, Anchor primer (SEQ ID NO: 2) and MHC-G1 primer (SEQ ID NO: 3) (S. T. Jones, et al., Biotechnology, 9, 88, 1991) were used.

The PCR solution (50 µl) used in this method comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM MgCl₂, 2.5 units of TaKaRa Taq (Takara Shuzo), 10 pmoles Anchor primer, and 1 µl of the reaction mixture of the cDNA to which MHC-G1 primer and Ampli FINDER Anchor primer had been ligated, over which 50 µl of mineral oil was layered. Thirty cycles of the PCR reaction was conducted using Thermal Cycler Model 480J (Perkin Elmer) and a temperature cycle of 94° C. for 45 sec.; 60° C. for 45 sec.; and 72° C. for 2 min.

(ii) Cloning of cDNA for #23-57-137-1 Antibody L Chain V Region

A DNA coding for L chain V region of the mouse monoclonal antibody against human PTHrP was cloned by the 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). This method was conducted using 5'-Ampli FINDER RACE Kit (Clontech) in accordance with the procedure by the manufacturer. In this method, oligo-dT primer was used as the primer for synthesizing a cDNA. About 2 µg of the above-mentioned mRNA (which was a template for cDNA synthesis) was mixed with oligo-dT primer. The resultant mixture was reacted with a reverse transcriptase at 52° C. for 30 min. to prepare a cDNA. The resultant was mixed with 6N NaOH to hydrolyze the RNA therein (at 65° C. for 30 min.). The resultant mixture was subjected to ethanol precipitation to isolate the cDNA as precipitates. The cDNA thus synthesized was ligated to the Ampli FINDER Anchor on its 5'-end by reacting with T4 RNA ligase at 37° C. for 6 hours and additionally at room temperature for 16 hours.

PCR primer MLC (SEQ ID NO: 4) was designed based on the conserved sequence of mouse L chain γ chain C region and then synthesized using 394 DNA/RNA Synthesizer (ABI). The PCR solution (100 µl) used for the synthesis of the primer comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM MgCl₂, 2.5 units of AmpliTaq (PERKIN ELMER), 50 pmoles of Anchor primer (SEQ ID NO: 2), and 1 µl of the reaction mixture of the cDNA to which MLC (SEQ ID NO: 4) and Ampli FINDER Anchor were ligated, over 50 µl of mineral oil was layered. Thirty-five cycles of the PCR reaction was conducted using Thermal Cycler Model 480J (Perkin Elmer) and a temperature cycle of 94° C. for 45 sec.; 60° C. for 45 sec.; and 72° C. for 2 min.

(3) Purification and Fragmentation of the PCR Product
Each of the DNA fragments amplified by the PCR methods described above was separated by agarose gel electro-

US 6,903,194 B1

29

phoresis using 3% Nu Sieve GTG agarose (FMC Bio. Products). For each of the H chain V region and the L chain V region, agarose gel fraction containing a DNA fragment of about 550 bp in length was excised from the gel, respectively. Each of the gel fractions obtained was subjected to purification of the DNA therefrom using GENECLEAN II Kit (BIO101) in accordance with the procedure by the manufacturer. The purified DNA was precipitated from the solution with ethanol and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. One μ l of the DNA solution thus prepared was digested with restriction enzyme XmaI (New England Biolabs) at 37° C. for 1 hour and additionally with restriction enzyme EcoRI (Takara Shuzo) at 37° C. for 1 hour. The digestion mixture was subjected to extraction with phenol and chloroform and then precipitation with ethanol to collect the DNA.

In this manner, obtained a DNA coding for the mouse H chain V region and a DNA coding for the mouse L chain V region, both which had an EcoRI recognition sequence on the 5'-end and an XmaI recognition sequence on the 3'-end thereof were obtained.

Each of the EcoRI-XmaI DNA fragments containing a DNA coding for the mouse H chain V region and a DNA coding for the mouse L chain V region, respectively, was reacted with pUC19 vector, which had been digested with EcoRI and XmaI, at 16° C. for 1 hour using DNA Ligation Kit ver.2 (Takara Shuzo) in accordance with the procedure by the manufacturer to ligate to each other. The ligation mixture (10 μ l) thus obtained was added to 100 μ l of a solution containing competent cells of *E. coli* JM 109 (Nippon Gene). The cell mixture was allowed to stand for 15 min. on ice, at 42° C. for 1 min. and further for 1 min. on ice. The resultant was mixed with 300 μ l of SOC culture medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) and then incubated at 37° C. for 30 min. The resultant cell solution was spread on a LB or 2xYT agar medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) supplemented with 100 or 50 μ g/ml of ampicillin, 0.1 mM of IPTG and 20 μ g/ml of X-gal and then incubated at 37° C. overnight. In this manner, *E. coli* transformants were prepared.

The transformants were cultured overnight in 2 ml of a LB or 2xYT medium containing 100 or 50 μ g/ml of ampicillin at 37° C. and then plasmid DNA was prepared from the cell fraction using Plasmid Extractor PI-100S (Kurabou) or QIAprep Spin Plasmid Kit (QIAGEN). The plasmid DNAs thus obtained were determined for their DNA sequences.

(4) Sequencing of cDNA Coding for V Region of Mouse Antibody

The sequence of the cDNA coding region carried on the plasmid was determined by DNA Sequencer 373A (ABI; Perkin-Elmer) using Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). This DNA sequence was determined by confirming the base sequence in the both orientations using primers, M13 Primer M4 (Takara Shuzo) (SEQ ID NO: 5) and M13 Primer RV (Takara Shuzo) (SEQ ID NO: 6).

The plasmids thus obtained, which contained a cDNA coding for the mouse H chain V region and a cDNA coding for the mouse L chain V region derived from hybridoma #23-57-137-1, were designated "MBC1H04" and "MBC1L24", respectively. The sequences (including the corresponding amino acid sequences) of the DNA coding for the H chain V region and the DNA coding for the L chain V region of mouse #23-57-137-1 antibody (respectively carried on plasmid MBC1H04 and plasmid MBC1L24)

30

were shown in SEQ. ID NOS: 57 and 65, respectively. Both of the polypeptides for the H chain V region fragment and for the L chain V region fragment were translated starting from the 58th base (which coding for glutamine) in the DNA sequences shown in SEQ ID NOS: 57 and 65. The amino acid sequences for the H chain V region and the L chain V region were shown in SEQ. ID NOS: 46 and 45, respectively.

The *E. coli* having plasmid MBC1H04 and the *E. coli* having plasmid MBC1L24 were designated "*Escherichia coli* JM109 (MBC1H04)" and "*Escherichia coli* JM109 (MBC1L24)", respectively. These *E. coli* strains have been deposited under the terms of the Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaragi-ken, Japan) on Aug. 15, 1996 under the Accession No. FERM BP-5628 for *Escherichia coli* JM109 (MBC1H04) and FERM BP-5627 for *Escherichia coli* JM109 (MBC1L24), respectively.

(5) Determination of CDR of Mouse Monoclonal Antibody #23-57-137-1 Against Human PTHrP

The general structures of the H chain V region and the L chain V region are similar to each other. That is, both structures have four framework regions ligated through three hypervariable regions [i.e., complementarity determining regions (CDRs)]. The amino acid sequences of the framework regions are relatively well conserved, while the amino acid sequences of the CDR regions exhibit an extremely high mutagenicity (Kabat, E. A. et al., "Sequence of Proteins of Immunological Interest", US Dept. Health and Human Services, 1983).

On the basis of the above-mentioned facts, the CDRs were determined by searching the homology of amino acid sequences of the mouse monoclonal antibody V region by reference to the Date Base of amino acid sequences for antibodies established by Kabat et al.

The amino acid sequences of CDRs 1-3 in the L chain V region are shown in SEQ ID Nos: 59-61, respectively, and the amino acid sequences of CDRs 1-3 in the H chain V region are shown in SEQ ID Nos: 62-64, respectively.

TABLE 2

V region	SEQ ID NO.	CDR1	CDR2	CDR3
H chain V region	57	31-35	50-66	99-107
L chain V region	65	23-34	50-60	93-105

EXAMPLE 2

Construction of Chimeric Antibody

- (1) Construction of Chimeric Antibody H Chain
(i) Construction of H Chain V region

The cloned cDNA coding for mouse H chain V region was modified by a PCR method to ligate it to an expression vector carrying the genomic DNA for the human H chain C region Cyl. The downstream-side primer MBC1-S1 (SEQ ID NO: 7) used was designed so as to be hybridizable to the DNA coding for the 51'-end region of the leader sequence of the V region and to have both a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and a HindIII-recognition sequence. The upstream-side primer, MBC1-a (SEQ ID NO: 8), used was designed so as to be hybridizable to the DNA coding for the 3'-end region of the J region and to have both a splice donor sequence and a BamHI-recognition sequence. The PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo) and a buffer appended thereto. The PCR solution (50 μ l) used comprised

US 6,903,194 B1

31

0.07 μ g of plasmid MBC1H04 as a template DNA, 50 pmol of MBC1-a and 50 pmol of MBC1-S1 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTP in the buffer, over which 50 μ l of mineral oil was layered. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min.; 55° C. for 1 min.; 72° C. for 2 min. The DNA fragments thus amplified by the PCR reaction were separated by agarose gel electrophoresis using 3% Nu Sieve GTG Agarose (FMC Bio. Products).

Then, an agarose gel fragment containing a DNA fragment of 437 bp in length was excised and the DNA fragment was purified therefrom using GENECLAN II Kit (Bio101) in accordance with an instruction included in the kit. The purified DNA was collected by ethanol precipitation, and then dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. One μ l of the resultant DNA solution was digested with restriction enzymes BamHI and HindIII (Takara Shuzo) at 37° C. for 1 hour. The digestion mixture was extracted with phenol and chloroform and then precipitated with ethanol to collect a DNA.

The obtained HindIII-BamHI DNA fragment, which contains a DNA coding for the mouse H chain V region, was subcloned into pUC19 vector which had been digested with HindIII and BamHI. The resultant plasmid was sequenced by DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV as primers, and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). The plasmid contained a DNA of correct base sequence coding for the mouse H chain V region derived from hybridoma #23-57-137-1 and had a HindIII-recognition sequence and a Kozak sequence on its 5'-end region and a BamHI-recognition sequence on its 3'-end region was designated "MBC1H/pUC19".

(ii) Construction of H Chain V Region to be Used for the Preparation of cDNA-Type of Mouse-Human Chimeric H Chain

The DNA coding for the mouse H chain V region constructed in the above step was modified by a PCR method to ligate it to a cDNA for the human H chain C region Cyl. The backward primer, MBC1HVS2, (SEQ ID NO: 9) used for the modification of H chain V region was designed so as to replace the second amino acid (i.e., asparagine) of the sequence coding for the front portion of the leader sequence of the V region with glycine and to have a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and the HindIII- and EcoRI-recognition sequences. The forward primer MBC1HVR2 (SEQ ID NO: 10) used for the modification of H chain V region was designed so as to be hybridizable to the DNA sequence coding for the 3'-end of the J region, to coding for the 5'-end region of the C region and to have Apal- and SmaI-recognition sequences.

The PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo) and a buffer appended thereto. The PCR solution (50 μ l) used comprised 0.6 μ g of plasmid MBC1H/pUC19 as a template DNA, 50 pmol of MBC1HVS2 and 50 pmol of MBC1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM of dNTP in the buffer, over which 50 μ l of mineral oil was layered. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min.; 55° C. for 1 min.; 72° C. for 1 min. The DNA fragments amplified by the PCR reaction were separated by agarose gel electrophoresis using 1% Sea Kem GTG Agarose (FMC Bio. Products). Then, an agarose gel fragment containing a DNA fragment of 456 bp in length was excised and the DNA fragment was purified therefrom using GENECLAN II Kit (Bio101) in accordance with the procedure by the manufacturer. The purified DNA fragments were precipitated with ethanol and then dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

32

One μ l of the resultant DNA solution was digested with restriction enzymes EcoRI and SmaI (Takara Shuzo) at 37° C. for 1 hour. The digestion mixture solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA. The obtained EcoRI-SmaI DNA fragments, which contains a DNA coding for the mouse H chain V region, was subcloned into pUC19 vector which had been prepared by digesting the plasmid with EcoRI and SmaI. The resultant plasmid was sequenced by DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV as primers, and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). The plasmid which contained a DNA of correct base sequence coding for the mouse H chain V region derived from hybridoma #23-57-137-1 and had a HindIII-recognition sequence and a Kozak sequence on its 5'-end region and Apal- and SmaI-recognition sequences on its 3'-end region was designated "MBC1H/pUC19".

(iii) Construction of Expression Vector for Chimeric Antibody H Chain

cDNA containing human antibody H chain C region Cyl was prepared as follows.

mRNA was prepared from a CHO cell into which both an expression vector DHFR- Δ E-RVh-PM-1-f (see WO/92/19759) coding for the genomic DNAs of the humanized PM1 antibody H chain V region and the human antibody H chain C region IgG1 and an expression vector RV1-PM1a (see WO/92/19759) coding for the genomic DNAs of the humanized PM1 antibody L chain V region and the human antibody L chain κ chain C region had been introduced. Using the mRNA obtained, was cloned a cDNA containing the humanized PM1 antibody H chain V region and the human antibody C region Cyl by a RT-PCR method, and then subcloned into plasmid pUC19 on the HindIII-BamHI site. The plasmid subcloned was determined for its DNA sequence and the plasmid which had a correct base sequence was designated "pRVh-PM1f-cDNA".

Expression vector DHFR- Δ E-RVh-PM-1-f which had deletions in the HindIII site between SV40 promoter and DHFR gene and the EcoRI site between EF-1 α promoter and the humanized PM1 antibody H chain V region was prepared for the construction of an expression vector for cDNA containing the humanized PM1 antibody H chain V region and the human antibody C region Cyl.

The plasmid pRVh-PM1f-cDNA obtained was digested with BamHI, blunt-ended with Klenow fragment, and further digested with Hind-III, to thereby obtain a blunt-ended HindIII-BamHI fragment. This blunt-ended HindIII-BamHI fragment was ligated to the above-mentioned HindIII site- and EcoRI site-deleted expression vector DHFR- Δ E-RVh-PM1-f which had been digested with HindIII and BamHI to construct expression vector RVh-PM1f-cDNA containing cDNAs coding for the humanized PM1 antibody H chain V region and the human antibody C region Cyl, respectively.

The expression vector RVh-PM1f-cDNA containing cDNAs coding for the humanized PM1 antibody H chain V region and the human antibody C region Cyl was digested with Apal and BamHI, and the DNA fragment containing the H chain C region was collected therefrom. The resultant DNA fragment was introduced into the above-mentioned plasmid MBC1H/pUC19 which had been digested with Apal and BamHI. The plasmid thus prepared was designated "MBC1HcDNA/pUC19". This plasmid is a plasmid contained cDNAs coding for the mouse antibody H chain V region and the human antibody C region Cyl, respectively, and having EcoRI- and HindIII-recognition sequences on its 5'-end and a BamHI-recognition sequence on its 3'-end.

US 6,903,194 B1

33

The plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to obtain a DNA coding for the chimeric antibody H chain. The resultant DNA fragment was introduced into expression vector pCOS1 which had been digested with EcoRI and BamHI. The expression vector for the chimeric antibody thus obtained was designated "MBC1HcDNA/pCOS1". Here, the expression vector pCOS1 was constructed using HEF-PMh-gyl (see WO/92/19759) by deleting therefrom the antibody gene by means of the digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo).

For preparing a plasmid for the expression in a CHO cell, the plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to obtain a DNA coding for the chimeric antibody H chain, which was then introduced into expression plasmid pCHO1 which had been digested with EcoRI and BamHI. The expression plasmid for the chimeric antibody thus obtained was designated "MBC1HcDNA/pCHO1". Here, the expression vector pCHO1 was constructed using DHFR- Δ E-rvH-PMI-I (see WO/92/19759) by deleting therefrom the antibody gene by means of the digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo).

(2) Construction of Human I Chain C Region

(i) Preparation of Cloning Vector

To construct pUC19 vector containing the human I chain C region, a HindIII site-deleted pUC19 vector was prepared. Two μ g of pUC19 vector was digested in 20 μ l of a reaction solution comprising 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8 U of HindIII (Takara Shuzo) at 37° C. for 1 hour. The resultant digestion mixture solution was extracted with phenol and chloroform and then was subjected to ethanol precipitation to collect the DNA of interest.

The DNA thus collected was reacted in 50 μ l of a reaction solution comprising 50 μ M Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 100 mM NaCl, 0.5 mM dNTP and 6U of Klenow fragment (GIBCO BRL) at room temperature for 20 min. to thereby render the ends of the DNA blunt. This reaction mixture was extracted with phenol and chloroform and then subjected to ethanol precipitation to collect the vector DNA.

The vector DNA thus collected was reacted in 10 μ l of a reaction solution comprising 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (v/v) polyethylene glycol-8000 and 0.5 U of T4 DNA ligase (GIBCO BRL) at 16° C. for 2 hours to cause self-ligation of the vector DNA. 5 μ l of the reaction solution was added to 100 μ l of a solution containing competent cells of *E. coli* strain JM109 (Nippon Gene) and the resultant solution was allowed to stand on ice for 30 min., at 42° C. for 1 min. and further on ice for 1 min. 500 μ l of SOC culture medium was added to the reaction solution, and then incubated at 37° C. for 1 hour. The resultant solution was plated on a 2xYT agar medium (containing 50 μ g/ml of ampicillin) which had been applied with X-gal and IPTG on its surface (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989), and then cultured at 37° C. overnight, thereby obtaining a transformant.

The transformant was cultured on a 2xYT medium containing 50 μ g/ml of ampicillin at 37° C. overnight. From the cell fraction of the culture medium, was purified a plasmid DNA using Plasmid Mini Kit (QIAGEN) in accordance with an instruction included in the kit. The purified plasmid was digested with HindIII. The plasmid which was confirmed to have a HindIII site-deletion was designated "pUC19 Δ HindIII".

34

(ii) Construction of DNA Coding for Human I Chain λ Chain C Region

The human antibody I chain λ chain C region has been known to have at least four isotypes including Mcg⁺Kc⁺Oz⁺, Mcg⁺Kc⁺Oz⁻, Mcg⁻Kc⁺Oz⁺ and Mcg⁻Kc⁺Oz⁻ (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987). The search was made for a human antibody I chain λ chain C region homologous to the #23-57-137-1 mouse I chain λ chain C region based on the EMBL data base. As a result, it was found that the isotype Mcg⁺Kc⁺Oz⁺ (Accession No. X57819) (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987) of the human antibody I chain λ chain exhibited the highest homology with the #23-57-137-1 mouse I chain λ chain C region and showed 64.4% homology in terms of amino acid sequence and 73.4% homology in terms of DNA sequence.

Then, the construction of the DNA coding for the human antibody I chain λ chain C region was conducted by a PCR method. Each of the following primers was synthesized using 394 DNA/RNA Synthesizer (ABI). The primers synthesized were HLAMB1 (SEQ ID NO: 11) and HLAMB3 (SEQ ID NO: 13) both having a sense DNA sequence and HLAMB2 (SEQ ID NO: 12) and HLAMB4 (SEQ ID NO: 14) both having an antisense DNA sequence, each primer containing a complementary sequence of 20-23 bp in length on the both ends.

External primers HLAMB5 (SEQ ID NO: 15) and HLAMBR (SEQ ID NO: 16) have a sequence complementary to the primers HLAMB1 and HLAMB4, respectively, and contain the EcoRI-, HindIII- and BlnI-recognition sequences and the EcoRI-recognition sequence, respectively. In the first PCR reaction, the HLAMB1-HLAMB2 and HLAMB3-HLAMB4 reactions were conducted. After the reactions were completed, both of the resultants were mixed with each other in equivalent quantities, and then assembled in the second PCR reaction. To the resultant reactant, were added the external primers HLAMB5 and HLAMBR. This reaction mixture was subjected to the third PCR reaction for amplifying the full length DNA.

The PCR reactions were conducted using TaKaRa Ex Taq (Takara Shuzo) in accordance with the procedure by the manufacturer. In the first PCR reaction, was used 100 μ l of either a reaction solution comprising 5 pmoles of HLAMB1, 0.5 pmole of HLAMB2 and 5U of TaKaRa Ex Taq (Takara Shuzo) or a reaction solution comprising 0.5 pmole of HLAMB3, 5 pmoles of HLAMB4 and 5U of TaKaRa Ex Taq (Takara Shuzo), over which 50 μ l of mineral oil was layered, and five cycles of the PCR reaction was conducted using a temperature cycle program of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min. In the second PCR reaction, was used a mixture of 50 μ l of each of the reaction solutions, over which 50 μ l of mineral oil was layered, and three cycles of the PCR reaction was conducted using a temperature cycle program of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min. In the third PCR reaction, the reaction solution to which 50 pmoles of each of the external primers HLAMB5 and HLAMBR were added was used, and thirty cycles of the PCR reaction was conducted using a temperature cycle program of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min.

The DNA fragment obtained by the third PCR reaction was subjected to electrophoresis using 3% low melting agarose gel (NuSieve GTG Agarose, FMC), and collected and purified from the gel using GENECLON II Kit (BIO101) in accordance with the procedure included in the kit.

The DNA fragment thus obtained was digested in 20 μ l of a reaction solution comprising 50 mM Tris-HCl (pH 7.5), 10

US 6,903,194 B1

35

mM $MgCl_{21}$, 1 mM DTT, 100 mM NaCl and 8U of EcoRI (Takara Shuzo) at 37° C. for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to thereby obtain the DNA. The DNA was collected and dissolved in 8 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

0.8 μ g of the plasmid pUC19 Δ HindIII was digested with EcoRI in the same manner as mentioned above. The digestion solution was extracted with phenol and chloroform, followed by ethanol precipitation, obtaining a digested plasmid pUC19 Δ HindIII. The digested plasmid thus obtained was reacted in 50 μ l of a reaction solution comprising 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$ and alkaline phosphatase (*E. coli* C75; Takara Shuzo) at 37° C. for 30 min. to dephosphorylate the plasmid (i.e., BAP-treatment). The reaction solution was subjected to phenol and chloroform extraction and ethanol precipitation to obtain the DNA. The DNA thus obtained was dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

One μ l of the BAP-treated plasmid pUC19 Δ HindIII thus prepared was mixed with 4 μ l of the DNA obtained by the above-mentioned PCR reaction to ligate to each other using DNA Ligation Kit Ver.2 (Takara Shuzo). The resultant plasmid was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured overnight in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

With respect to the plasmid described above, the cloned DNA was confirmed on its sequence. For determination the DNA sequence, 373A DNA Sequencer (ABI) and primers "M13 Primer M4" and "M13 Primer RV" (Takara Shuzo) were used. As a result, it was found that the cloned DNA had a deleted portion of 12 bp in length therein. The plasmid containing the DNA was designated " Δ C Δ pUC19". Then, for making up for the portion, primers, HCLMS (SEQ ID NO: 17) and HCLMR (SEQ ID NO: 18), were newly synthesized and a correct DNA was reconstructed by a PCR method.

The first PCR reaction was conducted, using the plasmid Δ C Δ pUC19 containing the deleted DNA as a template, and the primers HLAMBS and HCLMS or primers HCLMS and HLAMB4. Each of the PCR reaction products was purified respectively. In the second PCR reaction, the PCR products were assembled with each. To the resultant, were added external primers HLAMBS and HLAMB4, followed by the third PCR reaction for amplifying the full length DNA.

In the first PCR reaction, 100 μ l of a reaction solutions comprising 0.1 μ g Δ C Δ pUC19 as a template, either 50 pmoles of each of the primers HLAMBS and HCLMR or 50 pmoles of each of the primers HCLMS and HLAMB4, and 5 U of TaKaRa Ex Taq (Takara Shuzo) was used, over which 50 μ l of mineral oil was layered, and thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min.

The PCR products, HLAMBS-HCLMR (236 bp) and HCLMS-HLAMB4 (147 bp) were subjected to electrophoresis using 3% low melting agarose gel to isolate the DNA fragment. The DNA fragment was then collected and purified from the gel using GENECLAN II Kit (BIO101). In the second PCR reaction, 20 μ l of a reaction solution comprising 40 ng of the purified DNA fragments and 1U of TaKaRa Ex Taq (Takara Shuzo) were used, over which 25 μ l of mineral oil was layered, and five cycles of a temperature cycle of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min. was executed.

In the third PCR reaction, 100 μ l of a reaction solution comprising 2 μ l of the reaction solution obtained by the

36

second PCR reaction, 50 pmoles of each of external primers HLAMBS and HLAMB4 and 5U of TaKaRa Ex Taq (Takara Shuzo) were used, over which 50 μ l of mineral oil was layered, and thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min., thereby obtaining a DNA fragment of 357 bp in length (third PCR product). The DNA fragment was subjected to electrophoresis using 3% low melting agarose gel to isolate the DNA fragment. The resultant DNA fragment was collected and purified using GENECLAN Kit (BIO101).

An amount of 0.1 μ g of the DNA fragment thus obtained was digested with EcoRI, and then subcloned into a plasmid pUC19 Δ HindIII which had been treated with BAP. The resultant plasmid was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant thus prepared was cultured overnight in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The DNA sequence of the plasmid thus obtained was confirmed by using M13 Primer M4 and M13 Primer RV (Takara Shuzo) and determined 373A DNA Sequencer (ABI). The plasmid confirmed to have the correct DNA sequence without any deletion was designated " Δ C Δ pUC19". (iii) Construction of DNA Coding for Human L Chain, K Chain C Region

A DNA fragment coding for the L chain K chain C region was cloned from plasmid HEF-PM1k-gk (WO92/19759) by a PCR method. The forward primer HKAPS (SEQ ID NO: 19) was designed so as to contain the EcoRI and HindIII and BlnI-recognition sequences and the backward primer HKAPA (SEQ ID NO: 20) was designed so as to contain the EcoRI-recognition sequence, and both of them were synthesized using 394 DNA/RNA Synthesizer (ABI).

A PCR reaction was conducted using 100 μ l of a reaction solution comprising 0.1 μ g of plasmid HEF-PM1k-gk as a template, 50 pmoles of each of primers HKAPS and HKAPA and 5U of TaKaRa Ex Taq (Takara Shuzo), over which 50 μ l of mineral oil was layered. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 60° C. for 1 min. and 72° C. for 1 min., thereby obtaining a DNA fragment of 360 bp in length. The DNA fragment was isolated by electrophoresis using 3% low melting agarose, and then collected and purified using GENECLAN II Kit (BIO101).

The DNA fragment thus obtained was digested with EcoRI and then cloned into plasmid pUC19 Δ HindIII which had been treated with BAP. The resultant plasmid was introduced into a competent cell of *E. coli*, JM109, to obtain a transformant. The transformant thus obtained was cultured overnight in 2 ml of 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The purified plasmid DNA was sequenced using M13 Primer M4 and M13 Primer RV (Takara Shuzo) by means of 373A DNA Sequencer (ABI). The plasmid which was confirmed to have a correct base sequence was designated " Δ C Δ pUC19".

(3) Construction of Chimeric Antibody L Chain Expression Vector

Chimeric #23-57-137-1 antibody L chain expression vector was constructed. The DNA coding for #23-57-137-1 L chain V region was ligated to the HindIII and BlnI sites, present just front of the human antibody C region, of each of the plasmid Δ C Δ pUC19 and Δ C Δ pUC19, thereby obtaining pUC19 vectors each containing DNA coding for the chi-

US 6,903,194 B1

37

meric #23-57-137-1 antibody L chain V region and L chain λ or κ chain C region. Each of the resultant vectors was then digested with EcoRI to excise the DNA coding for the chimeric antibody L chain and the DNA was subcloned into HEF expression vector.

The DNA coding for #23-57-137-1 antibody L chain V region was cloned from plasmid MBC1L24 by a PCR method. The primers were individually synthesized using 394 DNA/RNA Synthesizer (ABI). The backward primer MBCCHL1 used (SEQ ID NO: 21) was designed to contain a HindIII-recognition sequence and a Kozak sequence (Kozak, M. et al., J. Mol. Biol. 196, 947-950, 1987) and the forward primer MBCCHL3 (SEQ ID NO: 22) was designed to contain BglII- and RcoRI-recognition sequences.

The PCR reaction was conducted using 100 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.1 μ g MBC1L24, 50 pmoles of each of primers MBCCHL1 and MBCCHL3 as primers and 1 μ l of AmpliTaq (PERKIN ELMER), over which 50 μ l of mineral oil was layered. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 45 sec., 60° C. for 45 sec. and 72° C. for 2 min.

The DNA fragment of 444 bp was electrophoresed using 3% low melting agarose gel, and collected and purified using GENECLAN Kit (BIO101). The purified DNA fragment was dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. One μ l of the PCR product was digested in 20 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 8U of HindIII (Takara Shuzo) and 8U of EcoRI (Takara Shuzo) at 37° C. for 1 hour. The digestion solution was extracted with phenol and chloroform, followed by ethanol precipitation to collect the DNA as precipitates. The DNA thus obtained was dissolved in 8 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

One μ g of plasmid pUC19 was digested with HindIII and EcoRI in the same manner as mentioned above, and then extracted with phenol and chloroform, followed by ethanol precipitation to collect the digested plasmid. The resultant was treated with BAP [i.e., an alkaline phosphatase (*E. coli* C75; Takara Shuzo)] and then extracted with phenol and chloroform, followed by ethanol precipitation, thereby obtain the DNA. The resultant DNA was dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

One μ l of the BAP treated plasmid pUC19 was mixed with 4 μ l of the above-mentioned PCR product to ligate each other using DNA Ligation Kit Ver. 2 (Takara Shuzo). The resultant plasmid was introduced into a competent cell of *E. coli*, JM109 (Nippon Gene), in the same manner as described above to form a transformant. The transformant was inoculated overnight on a 2xYT agar medium containing 50 μ g/ml of ampicillin at 37° C. The resultant transformant was then cultured overnight in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin at 37° C. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). After determining the DNA sequence, the plasmid confirming to have a correct DNA sequence was designated "CHL-pUC19".

One μ g of each of plasmids C λ /pUC19 and C κ /pUC19 was respectively digested in 20 μ l of a reaction solution comprising 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8 U of HindIII (Takara Shuzo) and 2U of BlnI (Takara Shuzo) at 37° C. for 1 hour. The digestion solution was extracted with phenol and chloroform, followed by ethanol precipitation, thereby obtaining a DNA.

38

The DNA was treated with BAP at 37° C. for 30 min. and then extracted with phenol and chloroform, followed by ethanol precipitation. The resultant was dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Eight μ g of the plasmid CHL/pUC19 which contained a DNA coding for #23-57-137-1 L chain V region was digested with HindIII and BlnI in the same manner as mentioned above. The DNA fragment of 409 bp in length thus obtained was electrophoresed using 3% low melting agarose gel and then collected and purified using GENECLAN II Kit (BIO101) from the gel. The DNA fragment was dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Four μ l of the L chain V region DNA was subcloned into 1 μ l of each of the BAP-treated plasmids C λ /pUC19 or C κ /pUC19, and then introduced into a competent cell of *E. coli*, JM109, to format a transformant. The transformant was cultured overnight in 3 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was isolated and purified using QIAprep Spin Plasmid Kit (QIAGEN). The plasmids thus prepared were designated "MBC1L(λ)/pUC19" and "MBC1L(κ)/pUC19", respectively.

Each of plasmids MBC1L(λ)/pUC19 and MBC1L(κ)/pUC19 was digested with EcoRI and then subjected to electrophoresis using 3% low melting agarose gel. A DNA fragment of 743 bp in length was isolated and purified from the gel using GENECLANII Kit (BIO101) and dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

An amount of 2.7 μ g of expression vector, plasmid HEF-PM1k-gk, was digested with EcoRI and then extracted with phenol and chloroform, followed by ethanol precipitation, thereby obtaining a DNA fragment. The DNA fragment was treated with BAP, and then subjected to electrophoresis using 1% low melting agarose gel. From the gel, a DNA fragment of 6561 bp in length was isolated and purified therefrom using GENECLANII Kit (BIO101). The DNA fragment was dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The HEF vector thus prepared was treated with BAP, and 2 μ l of the BAP-treated HEF vector was mixed with 3 μ l of the EcoRI fragments of plasmids MBC1L(λ)/pUC19 or MBC1L(κ)/pUC19, to ligate to each other. The ligation product was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The plasmid DNA thus purified was digested in 20 μ l of a reaction solution comprising 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8 U of HindIII (Takara Shuzo) and 2 U of PvuII (Takara Shuzo) at 37° C. for 1 hour. In this digestion reaction, it was assumed that if the above-mentioned DNA fragment was inserted into the vector in the correct orientation, a digestion fragment of 5104/2195 bp would be obtained, whereas if the above-mentioned DNA fragment was inserted into the vector in the reverse orientation, a digestion fragment of 4387/2926 bp would be obtained. Based on this assumption, the plasmids in which the fragment was inserted in a correct orientation were designated "MBC1L(λ)/neo" and "MBC1L(κ)/neo", respectively.

(4) Transfection of COS-7 Cells

The expression plasmids prepared above were each expressed transiently using COS-7 cells in order to evaluate

US 6,903,194 B1

39

the chimeric antibody on its binding with and neutralizing activities against antigen.

The transient expression of the chimeric antibody was conducted using a combination of either plasmids MBC1HcDNA/pCOS1 and MBC1L (λ)/neo or plasmids MBC1HcDNA/pCOS1 and MBC1L(k)/neo by means of electroporation using Gene Pulser (Bio Rad) to co-transfect each of these plasmid DNA combinations into COS-7 cells. That is, into 0.8 ml of a cell suspension in which COS-7 cells were suspended in PBS(-) in a concentration of 1×10^7 cells/ml, 10 μ g of each of the plasmid DNAs was added. The resultant solution was applied with pulses at an electrostatic capacity of 1,500V and 2μ F to cause electroporation. After 10 min. of recovery period at room temperature, the cells were suspended in a DMEM medium containing 2% Ultra Low IgG fetal calf serum (GIBCO) and then cultured using a 10 cm culture dish in a CO₂ incubator. After culturing for 72 hours, a culture supernatant was collected and centrifuged to remove cell debris and was provided as a sample for the ELISA assay.

In this procedure, the purification of the chimeric antibodies from the culture supernatant of COS-7 cells was conducted using AffiGel Protein A MAFSPL Kit (Bio Rad) in accordance with an instruction included in kit.

(5) ELISA Assay

(i) Determination of Antibody Concentration

An ELISA plate for determining antibody concentration was prepared as follows. Each of the wells of a 96-well plate for ELISA (Maxisorp, NUNC) was coated with 100 μ l of a solution comprising goat anti-human IgG antibody (TAGO) prepared in a coating buffer (0.1 M NaHCO₃, 0.02% Na₂S₂O₃) of a concentration of 1 μ g/ml and then blocked with 200 μ l of a dilution buffer (50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, 0.05% Tween20, 0.02% Na₂S₂O₃, 1% bovine serum albumin (BSA); pH 7.2). Into each well was added, a culture supernatant of the COS-7 cells in which the chimeric antibodies had been expressed or the purified chimeric antibodies at stepwise dilution. After incubating at room temperature for 1 hour and washing with PBS-Tween20, each well was added with 100 μ l of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After incubating at room temperature for 1 hour and washing with PBS-Tween20, each well was added with 1 mg/ml of a substrate solution ("Sigma104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured for absorbance at 405 nm using Microplate Reader (Bio Rad). As a standard for this determination, Hu IgG1 λ Purified (The Binding Site) was used.

(ii) Determination of antigen binding ability An ELISA plate for the determination of antigen binding ability was prepared as follows. Each of the wells of a 96-well plate for ELISA was coated with 100 μ l of a solution comprising human PTHrP (1-34) (Peptide Research Institute) prepared in a coating buffer of a concentration of 1 μ g/ml and then blocked with 200 μ l of a dilution buffer. Into each well was added, the culture supernatant of the COS-7 cells in which the chimeric antibodies had been expressed or the purified chimeric antibodies at stepwise dilution. After incubating at room temperature and washing with PBS-Tween20, each well was added with 100 μ l of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After incubating at room temperature and washing with PBS-Tween20, each well was added with 1 mg/ml of a substrate solution ("Sigma104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured for absorbance at 405 nm using Microplate Reader (Bio Rad).

As a result, it was found that the chimeric antibody had a binding ability against human PTHrP (1-34) and also had a

40

correct structure of the cloned mouse antibody V region (FIG. 4). It was also found that there was no difference in the binding ability against PTHrP (1-34) between the chimeric antibody in which the L chain C region has λ chain and those in which the L chain C region has k chain. Therefore, the L chain C region of the humanized antibody was constructed using the humanized antibody L chain λ chain.

(6) Establishment of CHO Stable Transformed Cell Line

For establishing a stable transformant for the chimeric antibody, the above-mentioned expression plasmid was introduced into a CHO cell (DXB11).

Establishment of a stable transformant for the chimeric antibody was conducted using combinations of expression plasmids for CHO cell, MBC1HcDNA/pCHO1 and MBC1L (λ)/neo or expression plasmids for CHO cell, MBC1HcDNA/pCHO1 and MBC1L(k)/neo. These combinations of plasmids were individually co-transfected into CHO cells by electroporation using Gene Pulser (Bio Rad). Each of the expression vectors was cleaved with restriction enzyme PvuII to obtain a linear DNA. The resultant DNA was collected by extraction with phenol and chloroform and subsequent precipitation with ethanol. The plasmid DNAs thus prepared were respectively subjected to electroporation. Ten μ g of each of the plasmid DNAs was added to 0.8 ml of a cell suspension containing CHO cells in PBS(-) in a concentration of 1×10^7 cells/ml. The resultant mixture was applied with pulses at an electrostatic capacity of 1,500V and 25 μ F. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in a MEM- α medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO). The resultant suspension was cultured using three 96-well plates (Falcon) in a CO₂ incubator. On the day after starting the cultivation, the medium was replaced by a selective medium [a MEM- α medium supplemented with 10% fetal calf serum (GIBCO) and 500 mg/ml of GENTAMICIN (G418Sulfate; GIBCO) without ribonucleoside or deoxyribonucleoside]. From the culture medium, cells into which the antibody gene was introduced were selected. After replacing the selective medium by a fresh one, before and after two weeks of cultivation, the cells were observed under a microscope. When a cell growth was observed, the cells were determined for the amount of antibodies produced by the above-mentioned ELISA assay. Among the cells, those which produced a larger amount of antibodies were selectively collected.

The scale up of the culture of the stable transformant for the established antibodies was conducted in a roller bottle using a MEM medium supplemented with 2% Ultra Low IgG fetal calf serum without ribonucleoside or deoxyribonucleoside. On day 3 and day 4 of the cultivation, the culture supernatant was collected and then filtered using a filter having a pore size of 0.2 μ m (Millipore) to remove cell debris therefrom.

Subsequently, the purification of the chimeric antibodies from the culture supernatant of the CHO cells was conducted using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with an instruction included within. The purified chimeric antibodies were provided as samples for the determination of neutralizing activity and for the examination of efficacy on hypercalcemic model animals. The concentration and the binding activity of the purified chimeric antibodies against antigen were determined by the same ELISA system.

US 6,903,194 B1

41

EXAMPLE 3

Construction of Humanized Antibody

(1) Construction of Humanized Antibody H Chain

(i) Construction of Humanized H Chain V Region

Humanized #23-57-137-1 antibody H chain was prepared by CDR-grafting technique by means of PCR method. For preparing a humanized #23-57-137-1 antibody H chain (version "a") having a FR derived from human antibody S31679 (NMRF-PDB; Cuisinier, A. M. et al., Eur. J. Immunol., 23, 110-118, 1993), the following six types of PCR primers were used: CDR-grafting primers: MBCH1GHP1 (SEQ ID NO: 23) and MBCH1GHP3 (SEQ ID NO: 24) (both containing a sense DNA sequence) and MBCH1GHP2 (SEQ ID NO: 25) and MBCH1GHP4 (SEQ ID NO: 26) (both containing an antisense DNA sequence), all of which containing a complementary sequence of 15-21 bp in length on both ends thereof; and external primers: MBCH1HVS1 (SEQ ID NO: 27) and MBCH1HVR1 (SEQ ID NO: 28), both having a homology with the CDR-grafting primers MBCH1GHP1 and MBCH1GHP4, respectively.

The CDR-grafting primers MBCH1GHP1, MBCH1GHP2, MBCH1GHP3 and MBCH1GHP4 were isolated using an urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) and extracted from the gel fraction by a crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) in the following manner.

One nmole of each of the CDR-grafting primers was isolated with 6% denatured polyacrylamide gel to obtain DNA fragments. From the resultant DNA fragments, those having a desired length was identified on a silica gel thin plate by irradiation of UV ray and then collected therefrom by a crush-and-soak method. The resultant was dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo). The reaction solution (100 μ l) used in the PCR reaction comprised 1 μ l of each of the above-mentioned CDR-grafting primers MBCH1GHP1, MBCH1GHP2, MBCH1GHP3 and MBCH1GHP4, 0.25 mM dNTP and 2.5U of TaKaRa Ex Taq in the buffer. Five cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 55° C. for 1 min. and 72° C. for 1 min. Into the resultant reaction mixture were added, both of the external primers MBCH1HVS1 and MBCH1HVR1 each in an amount of 50 pmoles. Using this reaction mixture, additional 30 cycles of the PCR reaction was conducted using the same temperature cycle. The DNA fragment thus amplified was isolated by agarose gel electrophoresis using 4% Nu Sieve GTG agarose (FMC Bio. Products).

An agarose fragment containing a DNA fragment of 421 bp in length was excised and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with an instruction included in the kit. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction mixture obtained was used for subcloning of the DNA fragment into plasmid pUC19 which had been prepared by digesting the plasmid with BamHI and HindIII; thereafter the base sequence of the resultant plasmid was determined. A plasmid having a correct sequence was designated "hMBCHv/pUC19".

(ii) Construction of H Chain V Region for Humanized H Chain cDNA

For the ligation to the cDNA of humanized H chain C region C γ 1, the DNA of the humanized H chain V region

42

constructed in the above step was modified by a PCR method. In this method, the backward primer MBCH1HVS2 used was designed so as to be hybridizable to the sequence coding for the 5'-end region of the leader sequence of the V region and to carry a Kozak consensus sequence (Kozak et al., J. Mol. Biol. 196, 947-950, 1987) and HindIII- and EcoRI-recognition sequences. The forward primer MBCH1HVR2 used for the modification of the DNA for the H chain V region was designed so as to be hybridizable to both the DNA sequence coding for the 3'-end region of the J region and the DNA sequence coding for the 5'-end region of the C region and to carry Apal- and SmaI-recognition sequences.

The PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo) and a buffer was used therewith. The reaction solution used for the PCR reaction comprised 0.4 μ g of hMBCHv/pUC19 as a DNA template, 50 pmoles of each of MBCH1HVS2 and MBCH1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTP in the buffer. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 55° C. for 1 min. and 72° C. for 1 min. The DNA fragment thus amplified by the PCR method was isolated by agarose gel electrophoresis using 3% Nu Sieve GTG agarose (FMC Bio. Products).

A DNA fragment of 456 bp in length was excised and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with an instruction included within. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction mixture thus obtained was used for subcloning of the DNA fragment into plasmid pUC19 which had been prepared by digesting the plasmid with EcoRI and SmaI; thereafter the DNA sequence of the resultant plasmid was determined. The plasmid DNA thus prepared which contains a DNA coding for the mouse H chain V region derived from the hybridoma #23-57-137-1 and also contains the EcoRI- and HindIII-recognition sequences and a Kozak sequence on the 5'-end and the Apal- and SmaI-recognition sequences on the 3'-end was designated "hMBCH1 Hv/pUC19".

(2) Construction of Expression Vector for Humanized Antibody H Chain

Plasmid RVh-PM1f-cDNA containing a cDNA sequence of hPM1 antibody H chain was digested with Apal and BamHI to obtain a DNA fragment containing a base sequence of the H chain C region. The DNA fragment was introduced into plasmid hMBCH1 Hv/pUC19 which had been prepared by digesting the plasmid with Apal and BamHI. The plasmid thus prepared was designated "hMBCH1cDNA/pUC19". This plasmid was a plasmid containing both a DNA coding for the humanized #23-57-137-1 antibody H chain V region and a DNA coding for the human H chain C region C γ 1 and to have EcoRI- and HindIII-recognition sequences on the 5'-end region and a BamHI-recognition sequence on the 3'-end region. The base sequence and the corresponding amino acid sequence of the humanized H chain version "a" contained in the plasmid hMBCH1cDNA/pUC19 are shown in SEQ ID NO: 58 and SEQ ID NO: 56, respectively.

The plasmid hMBCH1cDNA/pUC19 was digested with EcoRI and BamHI to obtain a DNA fragment containing a base sequence coding for the H chain. The DNA fragment was introduced into expression plasmid pCOS1 which had been prepared by digesting the plasmid with EcoRI and BamHI. The expression plasmid for a humanized antibody thus obtained was designated "hMBCH1cDNA/pCOS1".

To prepare a plasmid for expression in a CHO cell, plasmid hMBCH1 cDNA/pUC19 was digested with EcoRI

US 6,903,194 B1

43

and BamHI to obtain a DNA fragment containing a DNA coding for the H chain. The DNA fragment was introduced into expression vector pCHO1 which had been prepared by digesting the plasmid with EcoRI and BamHI. The expression plasmid for the humanized antibody thus obtained was designated "hMBC1L hCDNA/pCHO1".

(3) Construction of L Chain Hybrid Variable Region

(i) Preparation of FR1, 2/FR3, 4 Hybrid Antibody

A DNA coding for the L chain in which the FR regions were recombined with those from a humanized antibody and a mouse (chimeric) antibody was constructed and evaluated the regions on their suitability for humanization. In this step, a hybrid antibody comprising FR1 and FR2 both derived from a human antibody and FR3 and FR4 both derived from a mouse antibody was prepared by utilizing the AflIII restriction site present in CDR 2.

Ten μ g of each of plasmids MBC1L (λ)/neo and hMBC1L (λ)/neo was digested in 100 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 10 U of AflIII (Takara Shuzo) at 37° C. for 1 hour. The reaction solution was subjected to electrophoresis using 2% low melting agarose gel, and DNA fragments of 6282 bp in length (referred to as "cl") and 1022 bp in length (referred to as h1) were collected and purified from the gel using GENECLEANII Kit (BIO101).

One μ g of each of the cl and h1 fragments obtained was subjected to treatment with BAP. The DNA fragment was extracted with phenol and ethanol, collected by ethanol precipitation, dissolved in 10 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

One μ l of each of the BAP-treated cl and h1 DNA fragments were mixed with 4 μ l of the h2 and c2 DNA fragments, respectively, to ligate to each other (at 4° C. overnight). The ligation product was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The plasmid DNA purified was digested in 20 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 2U of ApaLI (Takara Shuzo), and 8U of BamHI (Takara Shuzo) or HindIII (Takara Shuzo) at 37° C. for 1 hour. In this step, the plasmid was identified based on the expectation that if the cl-h2 fragment was correctly ligated in the plasmid, this ligation would give an ApaLI-digestion fragment of 5560/1246/498 bp or a BamHI/HindIII-digestion fragment of 7134/269 bp.

The expression vector coding for the human FR1, 2/mouse FR3, 4 hybrid antibody L chain was designated "h/mMBC1L (λ)/neo". On the other hand, a clone for the h1-cl could not be obtained. Therefore, recombination on a pUC vector was conducted, followed by cloning to a HEF vector. Here, used as templates were plasmid hMBC1L λ /pUC19, which contained a DNA coding for a humanized antibody L chain V region having no amino acid replacements and plasmid hMBC1L λ /pUC19, which contained a DNA coding for a humanized antibody L chain V region having an amino acid replacement at the 91-position amino acid in FR3 (i.e., amino acid number 87 according to the definition by Kabat), of tyrosine, by isoleucine.

Ten μ l of each of plasmids MBC1L (λ)/pUC19, hMBC1L λ /pUC19 and hMBC1L λ /pUC19 was digested in 30 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA, 16U of HindIII and 4U of AflIII at 37° C. for

44

1 hour. The reaction solution was subjected to electrophoresis using 2% low melting agarose gel, and then collected and purified the following DNA fragments using GENECLEANII Kit (BIO101): a DNA fragment of 215 bp in length from plasmid MBC1L (λ)/pUC19 (referred to as "c2") or a DNA fragment of 3218 bp in length from each of plasmids hMBC1L λ /pUC19 and hMBC1L λ /pUC19MBC (referred to as "h1" and "h1", respectively).

Each of the h1' and h1" fragments was individually ligated to the c2' fragment and then introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). The resultant plasmid DNAs containing the h1' fragment and the h1" fragment were designated "m/hMBC1L λ /pUC19" and "m/hMBC1L λ /pUC19", respectively.

Each of the plasmids m/hMBC1L λ /pUC19 and m/hMBC1L λ /pUC19 was digested with EcoRI. The DNA fragment of 743 bp in length was electrophoresed using 2% low melting agarose gel, and then collected and purified from the gel using GENECLEANII Kit (BIO101). The resultant was dissolved in 20 μ l of a solution comprising 101 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Four μ l of the DNA fragment obtained was mixed with 1 μ l of the above-mentioned BAP-treated HEF vector to ligate to each other. The ligation product was introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant was cultured in 2 ml of a 2xYT medium supplemented with 50 μ g/ml of ampicillin. From the cell fraction, the plasmid DNA was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The plasmid DNA purified was digested in 20 μ l of a reaction solution comprising 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo) and 2U of PvuII (Takara Shuzo) at 37° C. for 1 hour. In this step, the plasmid DNA was identified based on the expectation that if the DNA fragment was inserted in the plasmid in a correct orientation, this digestion would give a digestion fragment of 5104/2195 bp, whereas if the DNA fragment is inserted in the plasmid in the reverse orientation, this digestion would give a digestion fragment of 4378/2926 bp. The plasmids thus obtained were expression vectors coding for mouse FR1, 2/human FR3, 4 hybrid antibody L-chain, which were designated expression vectors "m/hMBC1L λ /neo" and "m/hMBC1L λ /neo", respectively.

(ii) Preparation of FR1/FR2 Hybrid Antibody

An FR1/FR2 hybrid antibody was prepared in the same manner as mentioned above using a SnaBI restriction site present in CDR1.

Ten μ g of each of the plasmids MBC1L (λ)/neo and mMBC1L (λ)/neo was digested in 20 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 6 U of SnaBI (Takara Shuzo) at 37° C. for 1 hour. The resultant reaction solution was further digested in 50 μ l of a reaction solution comprising 20 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 1 mM DTT, 100 mM KCl, 0.01% (w/v) of BSA and 6U of PvuII at 37° C. for 1 hour.

The resultant reaction solution was subjected to electrophoresis using 1.5% low melting agarose gel, and then DNA fragments of 4955 bp and 2349 bp in length were collected and purified from the gel using GENECLEANII Kit (BIO101). The DNA fragments obtained from plasmid MBC1L (λ)/neo were designated "m1" (4955 bp) and "m2" (2349 bp) and the DNA fragments obtained from plasmid hMBC1L (λ)/neo were designated "h1" (4955 bp) and "h2" (2349 bp). Each of the DNA fragments obtained was

US 6,903,194 B1

45

dissolved in 40 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

One μ l of each of the hm1 and m1 fragments was ligated to 4 μ l of each of the hm2 and m2 fragments, respectively, and then introduced into a competent cell of *E. coli*, JM109, to form a transformant. The transformant obtained was cultured in 2 ml of a 2xYT medium containing 50 μ g/ml of ampicillin. From the cell fraction, the plasmid DNA was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The plasmid DNA purified was digested in 20 μ l of a reaction solution comprising 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT and either 8U of ApaI (Takara Shuzo) or 2U of ApaII (Takara Shuzo) at 37° C. for 1 hour.

The plasmids (m1-hm2 and hm1-m2) thus prepared were identified based on the expectation that if each of the DNA fragments is ligated in the plasmid in a correct orientation, the digestion of the plasmid (m1-hm2) with ApaI and Apa.I would give a fragment of 7304 bp and fragments of 5560/1246/498 bp, respectively, and the digestion of the plasmid (hm1-m2) with ApaI and Apa.I would give fragments of 6538/766 bp and a fragment of 3335/2025/1246/498 bp, respectively.

As expression vector coding for a human FR1/mouse FR2, 3, 4 hybrid antibody L chain was designated "hmmMBCILG(A)neo" and a expression vector coding for a mouse FR1/human FR2/mouse FR3, 4 hybrid antibody L chain was designated "hmmMBCILG(A)neo".

(4) Construction of Humanized Antibody L Chain

A humanized #23-57-137-1 antibody L chain was prepared by CDR-grafting technique by means of PCR method. That is, a humanized #23-57-137-1 antibody L chain (version "a") was prepared which contained FR1, FR2 and FR3 derived from human antibody HSU0868 (GEN-BANK, Delfos M. et al., Scand. J. Immunol., 39, 95-103, 1994) and FR4 derived from human antibody S25755 (NBRF-PDB) using the six types of PCR primers:

CDR-grafting primers, MBCILGP1 (SEQ ID NO: 29) and MBCILGP3 (SEQ ID NO: 30), both having a sense DNA sequence, CDR-grafting primers, MBCILGP2 (SEQ ID NO: 31) and MBCILGP4 (SEQ ID NO: 32), both having an antisense DNA sequence, all of which CDR-grafting primers having a complementary sequence of 15-21 bp on the both ends thereof; and external primers, MBCILVS1 (SEQ ID NO: 33) and MBCILVR1 (SEQ ID NO: 34), both having a homology with the CDR-grafting primers MBCILGP1 and MBCILGP4, respectively.

The CDR-grafting primers MBCILGP1, MBCILGP2, MBCILGP3 and MBCILGP4 were isolated using a urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) and extracted from the gel fraction by a crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989).

One nmole of each of the CDR-grafting primers was isolated with 6% denatured polyacrylamide gel. From the resultant, a DNA fragment having a desired length was identified on a silica gel thin plate by irradiation of UV ray and then collected therefrom by a crush-and-soak method. The resultant was dissolved in 20 μ l of a solution comprising 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo) with a buffer. The reaction solution (100 μ l) used in the PCR reaction comprised 1 μ l of each of the CDR-grafting primers MBCILGP1, MBCILGP2, MBCILGP3 and MBCILGP4, 0.25 mM dNTP, 2.5 U of TaKaRa Ex Taq in the buffer. Five cycles of the PCR

46

reaction was conducted using a temperature cycle of 94° C. for 1 min., 55° C. for 1 min. and 72° C. for 1 min. Into the resultant reaction mixture were added 50 pmoles of each of the external primers MBCILVS1 and MBCILVR1. Using this reaction mixture, additional thirty cycles of the PCR reaction was conducted using the same temperature cycle. The DNA fragment thus amplified was isolated by agarose gel electrophoresis using 3% Nu Sieve GTG agarose (FMC Bio. Products).

An agarose fragment containing a DNA fragment of 421 bp in length was excised and the DNA fragment was purified from the gel using GENECLANII Kit (BIO101) in accordance with the instruction included with the kit. The PCR reaction mixture thus prepared was used for subcloning of the DNA fragment into plasmid pUC19 which had been prepared by digesting the plasmid with BamHI and HindIII. The resultant plasmid was determined of its DNA sequence. The plasmid thus prepared was designated "hMBCIL/pUC19". In this plasmid, however, it was found that the 104-position amino acid (amino acid number 96 according to the determining by Kabat) of CDR4 was replaced with arginine. To correct this amino acid to tyrosine, primer MBCILGP10R (SEQ ID NO: 35) was designed and synthesized. Then the PCR reaction was conducted using TaKaRa Ex Taq (Takara Shuzo) with a buffer. The reaction solution (100 μ l) used in the PCR reaction comprised 0.6 μ g of the plasmid hMBCIL/pUC19 as a template DNA, 50 pmoles of each of the primers MBCILVS1 and MBCILGP10R, 2.5U of TaKaRa Ex Taq (Takara Shuzo) and 0.25 mM dNTP in the buffer, over which mineral oil was layered. Thirty cycles of the PCR reaction was conducted using a temperature cycle of 94° C. for 1 min., 55° C. for 1 min. and 72° C. for 1 min. The DNA fragment thus amplified by PCR method was isolated by agarose gel electrophoresis using 3% Nu Sieve GTG agarose (FMC Bio. Products).

A DNA fragment of 421 bp in length was excised and the DNA fragment was purified therefrom using GENECLANII Kit (BIO101) in accordance with an instruction included with the kit. The PCR reaction mixture thus prepared was used for subcloning of the DNA fragment into plasmid pUC19 which had been prepared by digesting the plasmid with BamHI and HindIII.

The plasmid was determined of its DNA sequence using M13 Primer M4 and M13 Primer RV. As a result, it was confirmed that the plasmid had a correct sequence. The plasmid was then digested with HindIII and B1nI and a DNA fragment of 416 bp was isolated there from by electrophoresis using 1% agarose gel. The DNA fragment was purified using GENECLANII Kit (BIO101) in accordance with an instruction included with the kit and then introduced into plasmid Cx/pUC which had been prepared by digesting the plasmid with HindIII and B1nI. The resultant plasmid was designated "hMBCIL La λ /pUC19". This plasmid was digested with EcoRI to obtain a DNA fragment coding for humanized L chain. The DNA fragment was introduced into plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EFl α promoter. The plasmid thus obtained was designated "hMBCIL La λ /pCOS1". The DNA sequence (including the corresponding amino acid sequence) of the humanized L chain version "a" is shown in SEQ ID NO: 66. The amino acid sequence of the version "a" is shown in SEQ ID NO: 47.

Version "b" was prepared using mutagenic introduction technique by a PCR method. The version "b" was designed so as to replace the 43-position amino acid, glycine, (amino acid number 43 according to the definition by Kabat) with proline and to replace the 49-position amino acid, lysine,

US 6,903,194 B1

47

(amino acid number 49 according to the definition by Kabat) with aspartic acid. The PCR reaction was conducted using plasmid hMBC1 L Δ /pUC19 as a template with a mutagenic primer MBC1LGP5R (SEQ ID NO: 36) and primer MBC1LVS1. The DNA fragment obtained was digested with BamHI and HindIII, and the digestion fragment was subcloned into the BamHI-HindIII site of pUC19. After sequencing, the plasmid DNA obtained was digested with HindIII and AflII, and the resultant digestion fragment was ligated to plasmid hMBC1 L Δ /pUC19 which had been prepared by digesting the plasmid with HindIII and AflII.

The plasmid thus obtained was designated "hMBC1 L Δ /pUC19". This plasmid DNA was digested with EcoRI to obtain a DNA fragment containing a DNA coding for the humanized L chain. The DNA fragment was introduced into plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EF1 α promoter. The plasmid thus obtained was designated "hMBC1L Δ /pCOS1".

Version "c" was prepared using mutagenic introduction technique by a PCR method. The version "c" was designed so as to replace the 84-position amino acid, serine, (amino acid number 80 according to the definition by Kabat) with proline. The PCR reaction was conducted using plasmid hMBC1 L Δ /pUC19 as a template with a mutagenic primer MBC1LGP6S (SEQ ID NO: 37) and primer M13 Primer RV. The DNA fragment obtained was digested with BamHI and HindIII and then subcloned into pUC19 which had been prepared by digesting the plasmid with BamHI and HindIII. After sequencing, the plasmid DNA obtained was digested with BstPI and Aor51HI, and the resultant DNA fragment was ligated to plasmid hMBC1L Δ /pUC19 which had been prepared by digesting the plasmid with BstPI and Aor51HI. The plasmid thus obtained was designated "hMBC1 L Δ /pUC19". This plasmid DNA was digested with EcoRI to obtain a sequence containing a sequence coding for the humanized L chain. The sequence was introduced into the EcoRI site of plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EF1 α promoter. The plasmid thus obtained was designated "hMBC1 L Δ /pCOS1".

Versions "d", "e" and "f" were also prepared using mutagenic introduction technique by a PCR method. Each of the versions "d", "e" and "f" was designed so as to replace the 91-position amino acid, tyrosine, (amino acid number 87 according to the definition by Kabat) with isoleucine in the versions "a", "b" and "c", respectively. For each of the versions "d", "e" and "f", a PCR reaction was conducted using each of plasmid hMBC1 L Δ /pCOS1 (for version "d"), hMBC1L Δ /pCOS1 (for version "e") and hMBC1 L Δ /pCOS1 (for version "f") as a template with a mutagenic primer MBC1LGP11R (SEQ ID NO: 38) and primer M-S1 (SEQ ID NO: 44). The DNA fragment thus obtained was digested with BamHI and HindIII and then subcloned into pUC19 which had been prepared by digesting pUC19 with BamHI and HindIII. After sequencing, the plasmid was digested with HindIII and BlnI, and the resultant digestion fragment was ligated to plasmid C Δ /pUC19 which had been prepared by digesting the plasmid with HindIII and BlnI.

The plasmids thus obtained were respectively designated "hMBC1 L Δ /pUC19", "hMBC1 L Δ /pUC19" and "hMBC1 L Δ /pUC19". Each of these plasmids was digested with EcoRI to obtain a DNA fragment containing a DNA coding for the humanized L chain. The DNA fragment was introduced into the EcoRI site of plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EF1 α promoter of the plasmid. The

48

plasmids thus obtained were respectively designated "hMBC1 L Δ /pCOS1", "hMBC1L Δ /pCOS1" and "hMBC1 L Δ /pCOS1".

Versions "g" and "h" were also prepared using mutagenic introduction technique by a PCR method. Each of the versions "g" and "h" was designed so as to replace the 36-position amino acid, histidine, (amino acid number 36 according to the definition by Kabat) with tyrosine in the versions "a" and "d", respectively. The PCR reaction was conducted with mutagenic primer MBC1LGP9R (SEQ ID NO: 39) and M13 Primer RV using plasmid hMBC1 L Δ /pUC19 as a template. The PCR product was subjected to an additional PCR reaction using M13 Primer M4 as a primer and plasmid hMBC1 L Δ /pUC19 as a template. The DNA fragment obtained was digested with HindIII and BlnI and then subcloned into plasmid C Δ /pUC19 which had been prepared by digesting the plasmid with HindIII and BlnI. Using this plasmid as a template, a PCR reaction was conducted using primers MBC1LGP13R (SEQ ID NO: 40) and MBC1LVS1. The PCR fragment obtained was digested with ApaI and HindIII and then introduced into each of plasmids hMBC1 L Δ /pUC19 and hMBC1 L Δ /pUC19 which had been prepared by digesting both plasmids with ApaI and HindIII. The plasmids obtained were determined of their DNA sequences. Plasmids which were confirmed to contain a correct sequence were designated "hMBC1 L Δ /pUC19" and "hMBC1 L Δ /pUC19", respectively. Each of these plasmids was digested with EcoRI to obtain a sequence containing a sequence coding for the humanized L chain. The sequence was introduced into the EcoRI site of plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EF1 α promoter. The plasmids thus obtained were respectively designated "hMBC1L Δ /pCOS1" and "hMBC1 L Δ /pCOS1".

Versions "i", "j", "k", "l", "m", "n" and "o" were also prepared using mutagenic introduction technique by a PCR method. The PCR reaction was conducted using a mutagenic primer MBC1LGP14S (SEQ ID NO: 41) and primer V1RV (Δ) (SEQ ID NO: 43) using plasmid hMBC1 L Δ /pUC19 as a template. The resultant DNA fragment was digested with ApaI and BlnI and then subcloned into plasmid hMBC1 L Δ /pUC19 which had been prepared by digesting the plasmid with ApaI and BlnI. The plasmid obtained was determined of its base sequence, and the clone into which the mutation corresponding to each of the versions was introduced, was selected. The plasmid thus obtained was designated "hMBC1 L Δ /pUC19 (x=i, j, k, l, m, n or o)". This plasmid was digested with EcoRI to obtain a sequence containing a sequence coding for the humanized L chain. The sequence was introduced into the EcoRI site of plasmid pCOS1 so that the initiation codon for the humanized L chain was located downstream from the EF1 α promoter. The plasmid thus obtained was designated "hMBC1 L Δ /pCOS1" (x=i, j, k, l, m, n or o). The DNA sequences (including the corresponding amino acid sequences) of the versions "j", "l", "m" and "o" are shown in SEQ ID NOS: 67, 68, 69 and 70, respectively. The amino acid sequences of these versions are shown in SEQ ID NOS: 48, 49, 50 and 51, respectively.

Versions "p", "q", "r", "s" and "t" are modified form of the versions "j", "l", "m", "n" and "o", respectively, in which the 87-position amino acid, tyrosine, is replaced with isoleucine. These versions were prepared by using the Aor51MI restriction site of FR3 for replacement of the version "h" with the version "i", "j", "m", "l" or "o" in the following manner. From expression plasmid hMBC1 L Δ /pCOS1 (x=i, j, m, l or o), an Aor51HI restriction fragment (514 bp)

US 6,903,194 B1

49

containing CDR3, a portion of FR3 and entire FR4 were deleted. To the deleted portion of the expression plasmid was ligated an Aor51H restriction fragment (514 bp) containing CDR3 and a portion of FR3 and entire FR4 so that the 91-position amino acid, tyrosine, (the amino acid number 87 according to the definition by Kabat) is replaced with isoleucine. The resultant plasmids were determined of their DNA sequence and the clone of each of the versions "i", "j", "m", "n", "o" and "p" in which 91-position amino acid, tyrosine, (the amino acid number 87 according to the definition by Kabat) was replaced with isoleucine was selected. The versions corresponding to the versions "i", "j", "m", "n" and "o" were designated versions "ip", "jq", "ms", "nt", and "or", respectively, and the plasmids for these versions were designated "hMBc1 L λ /pCOS1 (x=p, q, s, r or t). The DNA sequences (including the corresponding amino acids) of the versions "q", "r", "s" and "t" are shown in SEQ ID Nos: 71, 72, 73 and 74, respectively. The amino acid sequences of these versions are shown in SEQ ID Nos: 52, 53, 54 and 55, respectively.

Plasmid hMBc1 L λ /pCOS1 was digested with HindIII and EcoRI and then subcloned into plasmid pUC19 by digesting the plasmid with HindIII and EcoRI. The plasmid thus obtained was designated "hMBc1 L λ /pUC19.

The position of the replaced amino acids in each version of the humanized L chain is shown in Table 3 below.

TABLE 3

Position of replaced amino acid in sequence lists (amino acid number according to the definition by Kabat)							
Version	36	43	45	47	49	80	87
a							
b		P			D		
c						P	
d							I
e		P			D		I
f						P	I
g	Y						
h	Y					I	
i	Y		K		D		
j	Y		K				
k	Y		K	V			
l	Y		K	V	D		
m	Y				D		
n	Y		V				
o	Y		V	D			
p	Y		K				I
q	Y		K		D		I
r	Y				V		I
s	Y		K		V		I
t	Y				V		I

In Table 3 above, capital letters represent the following amino acids: Y: tyrosine; P: proline; K: lysine; V: valine; D: aspartic acid; and I: isoleucine.

E. coli strain containing plasmid hMBc1HcDNA/pUC19 and *E. coli* strain containing plasmid hMBc1 L λ /pUC19 were designated "Escherichia coli JM109 (hMBc1 HcDNA/pUC19)" and "Escherichia coli JM109 (hMBc1 L λ /pUC19)" respectively, which have been deposited under the terms of Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, (1-3, Higashi 1-chome, Tsukuba-shi, Ibaragi-ken, Japan) on Aug. 15, 1996 under the accession No. FERM BP-5629 for *Escherichia coli* JM109 (hMBc1 HcDNA/pUC19) and FERM BP-5630 for *Escherichia coli* JM109 (hMBc1 L λ /pUC19).

(5) Transfection into COS-7 Cell

For determining the antigen-binding activity and the neutralizing activity of the hybrid antibody and the human-

50

ized #23-57-137-1 antibody, the above-mentioned expression plasmids were expressed transiently in COS-7 cells. For the transient expression of the L chain hybrid antibody, each of the following combinations of plasmids was co-transfected into a COS-7 cell by electroporation using Gene Pulser (Bio Rad): hMBc1 HcDNA/pCOS1 and h/mMBc1L λ /neo; hMBc1 HcDNA/pCOS1 and m/hMBc1 L λ /neo; hMBc1 HcDNA/pCOS1 and m/hMBc1 L λ /neo; hMBc1 HcDNA/pCOS1 and h/mhMBc1L λ /neo; and hMBc1 HcDNA/pCOS1 and h/mhMBc1L λ /neo. That is, into 0.8 ml of a cell suspension in which COS-7 cells were suspended in PBS(-) in a concentration of 1×10^7 cells/ml, 10 μ g of each of the plasmid DNAs was added. The resultant solution was applied with pulses at an electrostatic capacity of 1,500V and 25 pF. After 10 min. of recovery period at room temperature, the cells treated by electroporation were suspended in a DMEM medium supplemented with 2% Ultra Low IgG fetal calf serum (GIBCO) and then cultured using a 10 cm culture dish in a CO₂ incubator. After culturing for 72 hours, a culture supernatant was collected and centrifuged to remove cell debris. The resultant was provided as a sample for the ELISA assay.

For the transient expression of the humanized #23-57-137-1 antibody, the plasmid combination of either hMBc1 HcDNA/pCOS1 or hMBc1 L λ /pCOS1 (x=a-i) was transfected into a COS-7 cell using Gene Pulser (Bio Rad) in the same manner as described for the hybrid antibody above. The culture supernatant obtained was provided as a sample for the ELISA assay.

Here, the purification of the hybrid antibody or the humanized antibody from the culture supernatant of COS-7 cell was conducted using AffiGel Protein A/MAPSH Kit (Bio Rad) in accordance with an instruction included in the kit.

(6) ELISA Assay

(i) Determination of Antibody Concentration

An ELISA plate for determining antibody concentration was prepared as follows. Each of the wells of a 96-well plate for ELISA (Maxisorp, NUNC) was coated with 100 μ l of a coating buffer (0.1 M NaHCO₃, 0.02% Na₂S₂O₃) supplemented with 1 μ g/ml of goat anti-human IgG antibody (TAGO) and then blocked with 200 μ l of a dilution buffer [50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, 0.05% Tween20, 0.02% Na₂S₂O₃, 1% bovine serum albumin (BSA); pH 7.2]. Into each of the wells was added a culture supernatant of the COS cells in which the hybrid antibody or the humanized antibody was expressed or a solution of the purified hybrid antibody or humanized antibody in stepwise dilution. After incubating at room temperature for 1 hour and washing with PBS-Tween20, 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO) was added to each of the wells. After incubating at room temperature for 1 hour and washing with PBS-Tween20, 1 mg/ml of a substrate solution ("Sigma104", p-nitrophenylphosphoric acid, SIGMA) was added to each of the wells. The solution was measured for absorbance at 405 nm using Microplate Reader (Bio Rad). As the standard for this determination of antibody concentration, Hu IgG1 λ Purified (The Binding Site) was used.

(ii) Determination of Antigen Binding Ability

An ELISA plate for determining antigen binding ability was prepared as follows. Each of the wells of a 96-well plate for ELISA (Maxisorp, NUNC) was coated with 100 μ l of a coating buffer supplemented with 1 μ g/ml of human PTHrP (1-34) and then blocked with 200 μ l of a dilution buffer. Thereafter, into each of the wells, was added a culture supernatant of the COS-7 cells in which the hybrid antibody

US 6,903,194 B1

51

or humanized antibody was expressed or a solution of the purified hybrid antibody or the purified humanized antibody in stepwise dilution. After incubating at room temperature and washing with PBS-Tween20, 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO) was added to each of the wells. After incubating at room temperature and washing with PBS-Tween20, 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA) was added to each of the wells. The solution was measured for absorbance at 405 nm using Microplate Reader (Bio Rad).

(7) Confirmation of Activities

(i) Evaluation of Humanized H Chain

It was found that an antibody comprising the humanized H chain version "a" and the chimeric L chain exhibited the same level of PTHrP-binding activity as that of the chimeric antibody (see FIG. 5). This result suggests that the humanization of the H chain V region is satisfactorily achieved by the version "a". Therefore, the humanized H chain version "a" was provided as a humanized antibody H chain in the following experiments.

(ii) Activity of Hybrid Antibody

(ii-a) FR1, 2/FR3, 4 Hybrid Antibody

When the L chain was h/mhMBC1Ld(λ), the antibody showed no antigen binding activity. However, when the L chain of the hybrid antibody was either mhMBC1 LaL or mhMBC1 LdL, the antibody showed the same level of antigen binding activity as that of the chimeric #23-57-137-1 antibody (FIG. 6). These results suggest that FR3 and FR4 are suitable for a humanized antibody but there exist amino acid residue(s) that need to be replaced in FR1 and FR2.

(ii-b) FR1/FR2 Hybrid Antibody

When the L chain of the hybrid antibody was mhMBC1L (λ), the antibody showed no antigen binding activity. However, when the L chain of the hybrid antibody was h/mhMBC1Ld(λ), the antibody showed the same level of antigen binding activity as that of the chimeric #23-57-137-1 antibody (FIG. 7). These results suggest that FR1 is suitable for a humanized antibody but there exist amino acid residue(s) that need to be replaced in FR2.

(iii) Activity of Humanized Antibody

The humanized antibody in which each of the versions "a" to "t" was used as the L chain was determined of its antigen-binding activity. As a result, it was found that the humanized antibodies having the L chain versions "j", "l", "m", "o", "q", "r", "s" and "t" exhibited the same level of PTHrP-binding activity as that of the chimeric antibody (FIGS. 8 to 11).

(8) Establishment of CHO Stable Production Cell Line

For establishing a stable transformant for humanized antibody, the above-mentioned expression plasmids were introduced into a CHO cell (DXB11).

The establishment of a stable transformant of the humanized antibody was conducted using each of the following combinations of plasmid as an expression vector for CHO cells: hMBC1HcDNA/pCOI and hMBC1 LmL/pCOSI; hMBC1 HcDNA/pCHOI and hMBC1 LqL/pCOSI; and hMBC1 HcDNA/pCHOI and hMBC1 LrL/pCOSI. The plasmids were co-transfected into a CHO cell by electroporation using Gene Pulser (Bio Rad). Subsequently, each of the expression vectors was cleaved with restriction enzyme PvuI to obtain a linear DNA. The resultant DNA was extracted with phenol and chloroform and then precipitated with ethanol. The DNAs thus prepared were respectively subjected to electroporation as follows. That is, 10 μ g of each of the plasmid DNAs was added to 0.8 ml of a cell suspension containing CHO cells in PBS(-) in a concentra-

52

tion of 1×10^7 cells/ml. The resultant mixture was applied with pulses at an electrostatic capacity of 1,500V and 25 μ F. After 10 min. of recovery period at room temperature, the cells thus treated were suspended in a MEM- α medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) and then cultured in a CO₂ incubator using 96-well plates (Falcon). On the day after starting the cultivation, the medium was replaced by a MEM- α selective medium supplemented with 10% fetal calf serum (GIBCO) and 500 mg/ml of GENETICIN (G418Sulfate; GIBCO) but containing no ribonucleoside or deoxyribonucleoside. From the culture medium, cells into which the antibody gene was introduced were selected. After replacing the culture medium by a fresh one, before and after two weeks of cultivation, the cells were observed microscopically. When a satisfactory cell growth was observed, the cells were determined for the amount of antibodies produced by an ELISA assay conventionally used for determining antibody concentration as described above. Among the cells, those which produced a larger amount of antibodies were selectively collected.

The scale up of the culture of the stable transformant for the antibodies thus established was conducted in a roller bottle using a MEM- α medium supplemented with 2% Ultra Low IgG fetal calf serum without ribonucleoside or deoxyribonucleoside. On each of day 3 and day 4 after the cultivation, the culture supernatant was collected and filtered using a 0.2 μ m filter having (Millipore) to remove cell debris therefrom. The purification of the humanized antibodies from the culture supernatant of the CHO cells was conducted using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with an instruction included. The humanized antibodies were provided as a sample for the determination of neutralizing activity and examination of pharmacological efficacy on hypercalcemic model animals. The concentration and the antigen-binding activity of the purified humanized antibodies were determined by the ELISA system as mentioned above.

EXAMPLE 4

Determination of Neutralizing activity

The determination of neutralizing activity of the mouse antibody, the chimeric antibody and the humanized antibody was conducted using rat myeloma cell line ROS17/2.8-5 cells. The ROS17/2.8-5 cells were cultured in Ham'S F-12 medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) using a CO₂ incubator. The ROS17/2.8-5 cells were inoculated in each of the wells of a 96-well plate in a concentration of 10^5 cells/100 μ l/well and cultured for 1 day. The culture medium was replaced with Ham'S F-12 medium (GIBCO) supplemented with 4 mM Hydrocortisone and 10% fetal calf serum. After culturing for three to four days, the cultured cells were washed with 260 μ l of Ham'S F-12 medium (GIBCO), and then 80 μ l of Ham'S F-12 medium supplemented with 1 mM isobutyl-1-methylxanthine (IBMX, SIGMA), 10% fetal calf serum and 10 mM HEPES was added thereto. The resultant mixture was incubated at 37° C. for 30 min.

The mouse antibody, the chimeric antibody and the humanized antibody to be tested for neutralizing activity were previously diluted stepwise in the following groups: [10 μ g/ml, 3.3 μ g/ml, 1.1 μ g/ml and 0.37 μ g/ml], [-10 μ g/ml, 2 μ g/ml, 0.5 μ g/ml and 0.01 μ g/ml] and [10 μ g/ml, 5 μ g/ml, 1.25 μ g/ml, 0.63 μ g/ml and 0.31 μ g/ml]. Each of the diluted antibody sample solution was mixed with an equivalent

US 6,903,194 B1

53

amount of 4 ng/ml of PTHrP (1-34). Eighty μ l of the resultant mixture solution was added into each well. The final concentration of each antibody became a quarter of the concentration of the above-mentioned antibody, and therefore the concentration of PTHrP (1-34) became 1 ng/ml. Ten minutes after the treatment at room temperature, the culture supernatant was removed and the residue was washed with PBS three times. From the resultant, cAMP in the cells was extracted with 10 μ l of a 0.3% HCl-95% ethanol and then extracted with a water jet aspirator to remove the HCl-ethanol. The residue was dissolved in 120 μ l of EIA buffer attached to cAMP EIA Kit (CAYMAN CHEMICAL'S) to extract the cAMP therefrom. The cAMP level was determined using cAMP EIA Kit (CAYMAN CHEMICAL'S) in accordance with an instruction included within. As a result, it was found that, among the humanized antibodies having L chain versions showing the same level of antigen-binding activity as that of the chimeric antibody, those having L chain versions "q", "r", "s" and "t" in which the 91-position tyrosine was replaced with isoleucine exhibited the closest neutralizing activity to that of the chimeric antibody, and especially those having a L chain version "q" exhibited the strongest neutralizing activity (FIGS. 12 to 14)

EXAMPLE 5

Examination of Pharmacological Efficacy on Hypercalcemic Model Animals (1)

Using a hypercalcemic model animal (a human tumor transplanted nude mouse), a chimeric antibody and humanized antibodies individually having L chain versions "m", "r" and "q" against PTHrP were examined for their therapeutic efficacy on hypercalcemia.

As a hypercalcemic model animal, was used a nude mouse which had been transplanted with human pancreatic cancer PAN-7 [purchased from the Central Institute for Experimental Animals]. It has been known that a nude mouse which has been transplanted with human pancreatic cancer PAN-7 exhibits an increased calcium concentration in blood as increasing the tumor volume and develops hypercalcemia which is associated with, for example, decrease in body weight and spontaneous activity. In this example, therapeutic effect of the chimeric antibody and the humanized antibody of the invention on hypercalcemia induced by the human pancreatic cancer PAN-7 was examined by the measurement of body weight and calcium concentration in blood of the test animal.

The passage of the human pancreatic cancer PAN-7 was conducted using BALB/c-nu/nude mice (Nippon Charles River) in vivo. For the evaluation of pharmacological efficacy, 5-weeks-old male BALB/c-nu/nude mice (Nippon Charles River) were purchased and then acclimatized them for 1 week, and the mice of 6-weeks-old thus prepared were used for the evaluation. The hypercalcemic model mice were prepared and divided into groups in the following manner. The human pancreatic cancer PAN-7 passed was excised and then finely cut into 3 mm cube of blocks. The resultant tumor blocks were subcutaneously transplanted under the skin flap of the mice at one piece per mouse. Two or three weeks after the transplantation, when it was confirmed that the tumor volume in each of the mice became satisfactorily large, the mice were divided into groups so that tumor volume, calcium concentration in blood and body weights of the mice of the individual groups were averaged, and the mice were used as the hypercalcemic model animals.

The examination of therapeutic efficacy on hypercalcemia was conducted as follows. A single dose of the chimeric

54

antibody or the humanized antibody having a L chain version "m" or "r" against PTHrP was administered to each of the above-mentioned hypercalcemic model mice via tail vein in a dose amount of 10 or 30 μ g per mouse. A single dose of the humanized antibody having a L chain version "q" was administered to each of the above-mentioned hypercalcemic model mice via tail vein in a dose amount of 20 or 60 μ g per mouse. On day 1, day 4, day 7 and day 11 after the administration, each of the mice was measured for the calcium concentration in blood and measured for the body weight to evaluate the pharmacological efficacy of the antibodies. The tumor volume was determined by measuring the major diameter (a mm) and the minor diameter (b mm) of the tumor and calculating using the both measured values according to Galant's equation [$ab^2/2$]. The calcium concentration in blood was determined as ionized calcium concentration in whole blood by drawing blood from each of the mice via the orbit using a hematocrit tube and applying the blood to 643 Automatic Ca^{++} /pH Analyzer (CIBA-CORNING).

As a result, it was found that the administration of the chimeric antibody and the humanized antibodies each having the L chain versions "m", "r" and "q" leads to a rapid improvement in change of body weight and calcium concentration in blood and a retention of improvement for a prolonged period of time for a subject. This result showed that the chimeric antibody and the humanized antibodies of the present invention are useful for treating malignant tumor-associated hypercalcemia (see FIGS. 15 and 16).

EXAMPLE 6

Examination of Pharmacological Efficacy on Hypercalcemia Model Animals (2)

Using a hypercalcemic model animal (a human tumor transplanted nude mouse), a chimeric antibody and a humanized antibody having L chain version "q" against PTHrP were examined for their therapeutic efficacy on hypercalcemia as follows.

The examination for the therapeutic efficacy on hypercalcemia was conducted as follows. A single dose of the chimeric antibody or the humanized antibody having a L chain version "q" against PTHrP was administered to each of the above-mentioned hypercalcemia model mice via tail vein in a dose amount of 10 or 30 μ g per mouse. On day 1, day 3, day 7 and day 11 after the administration, each of the mice was determined for the calcium concentration in blood and measured for the body weight to evaluate the pharmacological efficacy of the antibodies. The calcium concentration in blood was determined as ionized calcium concentration in whole blood by drawing blood from each of the mice via the orbit using a hematocrit tube and applying the blood to 643 Automatic Ca^{++} /pH Analyzer (CIBA-CORNING).

As a result, in the hypercalcemia model animal carrying human pancreatic cancer PAN-7, the administration of the chimeric antibody and the humanized antibody having the L chain version "q" leads to a rapid improvement with respect to body weight and calcium concentration in blood and to a retention of improvement for a prolonged time period for a subject. This result suggests that the chimeric antibody and the humanized antibody of the present invention are useful agent for treating malignant tumor-associated hypercalcemia (see FIG. 17).

EXAMPLE 7

Examination of Pharmacological Efficacy on Hypercalcemia Model Animals (3)

Using a hypercalcemia model animal (a human lung cancer LC-6 transplanted nude mouse), a chimeric antibody

US 6,903,194 B1

55

and a humanized antibody having L chain version "q" against PTHrP were examined for their therapeutic efficacy on hypercalcemia.

In this experiment, as the hypercalcemia model animal, a nude mouse into which human lung cancer LC-6 (purchased from the Central Institute for Experimental Animals) was transplanted was used. It has been known that a nude mouse into which human lung cancer LC-6 is transplanted tends to show an increased calcium concentration in blood with increased tumor volume and develops hypercalcemia associated with decrease in body weight and spontaneous activity.

In this example, therapeutic efficacy of the chimeric antibody and the humanized antibody of the invention on hypercalcemia induced by the human lung cancer LC-6 was examined by the measurement of body weight and calcium concentration in blood of the test animal.

The passage of the human lung cancer strain LC-6 was conducted using BALB/c-nu/nude mice (Nippon Charles River) in vivo. For the evaluation of pharmacological efficacy, 5-weeks-old male BALB/c-nu/nude mice (Nippon Charles River) were purchased and then acclimated for 1 week, and the mice of 6-weeks-old were used.

The hypercalcemia model mice were prepared and divided into groups in the following manner. The human lung cancer LC-6 was excised and then finely cut into 3 mm cube of blocks. The resultant tumor blocks were subcutaneously transplanted under the skin flap of the mice at one piece per mouse. Two or three weeks after the transplantation, when it was confirmed that the tumor volume in each of the mice had become satisfactorily large, the mice were divided into groups so that tumor volume, calcium concentration in blood and body weight of the mice of the individual groups were averaged, and the mice were used as the hypercalcemia model animals.

The examination of therapeutic efficacy on hypercalcemia was conducted as follows. A single dose of the chimeric antibody or the humanized antibody having a L chain version "q" against PTHrP was administered to each of the above-mentioned hypercalcemia model mice via tail vein in a dose amount of 10 or 30 μ g per mouse. On day 1, day 3, day 6 and day 10 after the administration, each of the mice was measured for the calcium concentration in blood and measured for the body weight to evaluate the pharmacological efficacy of the antibodies. The calcium concentration in blood was determined as ionized calcium concentration in whole blood by drawing blood from each of the mice via the orbit using a hematocrit tube and applying the blood to 643 Automatic Ca²⁺/pH Analyzer (CIBA-CORNING).

As a result, it was found that in the hypercalcemia model animal carrying human lung cancer LC-6, the administration of the chimeric antibody and the humanized antibody having the L chain version "q" leads to a rapid improvement with respect to body weight and calcium concentration in blood and to a retention of improvement for a prolonged time period for a subject. This result suggests that the chimeric antibody and the humanized antibody of the present invention are useful agent for treating malignant tumor-associated hypercalcemia (see FIG. 18).

EXAMPLE 8

Kinetic Analysis of Interaction between PTHrP and anti-PTHrP Antibody Using BIACORE

In this experiment, kinetic analysis of the antigen-antibody interaction using BIACORE was conducted.

56

PTHrP(1-34+Cys) was used as an antigen and adsorbed onto the sensor tip specifically for C-terminals. Purified antibodies of various concentrations were used as an analyte. From the sensogram obtained, kinetics parameters (binding rate constant "k_{ass}" and dissociation rate constant "k_{diss}") were calculated. With respect to the kinetic analysis, literature "Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system", Karlsson, R. et al., (1991), J. Immunol. Methods 145, p.229-240, was used for reference.

(1) Immobilization of PTHrP(1-34+C) Onto the Sensor Tip PTHrP (1-34+C) Was Adsorbed Onto Sensor Tip CM5 (Pharmacia).

As a running buffer, HBS (10 mM HEPES, pH 7.4; 0.15 M NaCl; 3.4 mM EDTA; 0.005% Surfactant P20) at a flow rate of 5 μ l/min. was employed. The carboxyl groups of calyxomethylidextran on the sensor tip CM5 were activated by an injection of 100 μ l of 0.05M N-hydroxysuccinimide (NHS)/0.2M N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and an injection of 100 μ l of 80 mM 2-(2-pyridyldithio)ethanamine (PDEA)/0.1 M borate buffer (pH 8.5), and an additional injection of 10 μ l of 5 μ g/ml PTHrP(1-34+C)/10 mM sodium acetate buffer (pH 5.0) to adsorb onto the C-terminals of PTHrP(1-34+C) specifically for Cys residues. Subsequently, an injection of 100 μ l of 50 mM (L)-cysteinyl/1M NaCl/0.1 M sodium formate buffer (pH 4.3) was conducted to block excessively activated groups. Subsequently, an injection of 10 μ l of 0.1M glycine-HCl buffer (pH 2.5) and 10 μ l of 10 mM HCl was conducted to wash the substances having non-covalent bonding. The amount of the PTHrP(1-34+C) thus immunized was 226.4 RU (resonance units) (FIG. 19).

(2) Interaction Between Immobilized PTHrP(1-34+C) and Purified Mouse Anti-PTHrP Antibody

As a running buffer, HBS at a flow rate of 20 μ l/min. was used. The antibody producing hybridomas were injected into the abdominal cavity of a Balb/c mouse and after a couple of weeks, the ascites were collected and applied on protein A column to purify antibodies. The purified #23-57-137-1 antibody was designated "MBC" and the purified 3F5 antibody was designated "3F5". These antibodies were diluted with HBS in a series of concentrations of 1.25, 2.5, 5, 10 and 20 μ g/ml.

In the analysis, 40 μ l of the antibody solution was injected for 2 min. to give a binding phase, and then HBS was injected for 2 min. to give a dissociation phase. After the dissociation was completed, 10 μ l of 10 mM HCl was injected to recover the sensor tip. The analysis was conducted by using this binding-dissociation-recovering as one cycle and injecting various antibody solutions to obtain a sensogram.

(3) Interaction Between Immobilized PTHrP(1-34+C) and Purified Humanized Anti-PTHrP Antibody

As a running buffer, HBS at a flow rate of 20 μ l/min. was used. The antibody was produced by CHO cells and purified using protein A column. The purified chimeric antibody was designated "chMBC", and the purified humanized antibodies of versions m and q were designated "hMBCm" and "hMBCq", respectively. These antibodies were diluted with HBS in a series of concentrations of 1.25, 2.5, 5, 10 and 20 μ g/ml.

In the analysis, 40 μ l of the antibody solution was injected for 2 min. to give a binding phase, and then HBS was injected for 2 min. to give a dissociation phase. After the dissociation was completed, 10 μ l of 10 mM HCl was injected to recover the sensor tip. The analysis was conducted by using a binding-dissociation-recovering as one cycle and by injecting various antibody solutions to obtain a sensogram.

US 6,903,194 B1

57

(4) Kinetic Analysis of the Interaction

The date file of interest was read and a comparison of the reaction patterns was conducted by overlaying the reaction regions of interest (FIGS. 20-24). In each of FIGS. 20-24, lines sequentially indicate from the top the data for the antibody concentrations 1.25, 2.5, 5, 10 and 20 $\mu\text{g/ml}$. Further, kinetic analysis of the interaction was conducted using an analysis software specifically designed for BIA-CORE "BIAevaluation 2.1" (Pharmacia) which is capable of calculating the kinetics parameters (binding rate constant "kass" and dissociation rate constant "kdiss") by curve cutting (Tables 4 and 5).

TABLE 4

Kinetics parameters of MBC and 3F5

	MBC	3F5
Kdiss [1/s]	7.38×10^{-6}	1.22×10^{-2}
Kass [1/Ms]	7.23×10^6	6.55×10^9
KD [M]	1.02×10^{-10}	1.86×10^{-6}

TABLE 5

Kinetics parameters of chimeric and humanized antibodies

	chH-ch λ	hMBCm	hMBCq
Kdiss [1/s] ($\times 10^{-4}$)	1.66	3.16	2.32
Kass [1/Ms] ($\times 10^{-4}$)	1.24	0.883	1.03
KD [M] ($\times 10^{-10}$)	1.34	3.58	2.25

In this experiment, for determining the binding rate constant, analysis model type 4 (BIAevaluation 2.1 Software Handbook, A1-A5) was used.

EXAMPLE 9

Suppression of Excretion of Phosphorus in a Model of Malignant Tumor-Associated Hypercalcemia

Malignant tumor-associated hypercalcemia (HHM) is a disease caused by the presence of PTHrP and it has been known that PTHrP accelerates bone resorption and calcium resorption in kidney and reniferous tubule, resulting in the development of hypercalcemia. On the other hand, with respect to phosphorus, PTHrP suppress the resorption of phosphorus in the kidney and reniferous tubule, resulting in the development of eliminant action, and therefore clinical HHM patients often develop hypophosphatemia. Here, the effect of humanized anti-PTHrP antibody on excretion of phosphorus in the kidney was examined using malignant tumor-associated hypercalcemia model rats.

As a model animal, a nude rat into which human lung cancer LC-6 (purchased from the Central Institute for Experimental Animals) was transplanted was used. It has been known that a nude rat into which human lung cancer LC-6 was subcutaneously transplanted tends to show an increased calcium concentration in blood was the increase of the tumor volume and, as a result, the rat develops hypercalcemia which is associated with, for example, decrease in body weight and spontaneous activity. Using this model animal, the effect of the humanized anti-PTHrP antibody of the invention on phosphate excretion in the kidney was examined by a renal clearance method based on the below-mentioned fractional excretion of phosphate.

The passage of the human lung cancer LC-6 was conducted using BALB/c-nu nu nude mice (Nippon Kurea) in

58

vivo. For the evaluation of pharmacological efficacy, 5-weeks-old male F344N/Jcl-nu nude rats (Nippon Kurea) were purchased and then acclimatized them for 1 week, and the rats of 6-weeks-old were used.

The malignant tumor-associated hypercalcemia model animals were prepared as follows. The human lung cancer LC-6 tumor passed was excised and then finely cut into 3 mm cube of blocks. The resultant tumor blocks were subcutaneously transplanted under the skin flap of the rats at one piece per rat. About thirty days after the transplantation, when it was confirmed that the tumor volume in each of the rats became satisfactorily large (3000 mm^3), the rats to be provided as the malignant tumor-associated hypercalcemia model animals were selected based on calcium concentration in blood and body weight.

The examination of phosphate excretion by a renal clearance method was conducted in the following manner.

(1) Renal Clearance Method

A malignant tumor-associated hypercalcemia model animal was anesthetized with pentobarbital (Nembutal, Dai-
 19 nippon Pharmaceutical Co., Ltd.), fixed supinely onto a incubation mat maintained at 37°C, and inserted a cannula (a polyethylene tube, PES0, Nippon Beckton Dickinson) to its bladder to collect urine. Subsequently, the model animal was inserted a cannula for infusion (a polyethylene tube, PE10, Nippon Beckton Dickinson) to its femoral vein, and then an infusion solution (0.7% inulin, 5% mannitol, 0.2% pentobarbital and 0.9% sodium chloride) was introduced into the model animal through the cannula at a flow rate of 2 ml/hr using an infusion pump (Icrufusion syringe pump; STC-525; TERUMO) to infuse the model animal. After equilibrating for 50 min., urine was collected through the cannula for five times at 20 min. intervals (i.e., from period-1 to period-5) to give urine samples. At the intermediate point of time during each urine collection procedure, approximately 0.25 ml of blood samples from the right cervical vein of the model animal were collected using a heparin-treated injection syringe.

(2) Administration of Antibody

During the above-mentioned clearance test, at the point of time where the collection of urine of period-2 was just started, a humanized anti-PTHrP antibody was administered intravenously to the animal in a dose amount of 1 mg/ml/kg.

(3) Determination of Concentration of Inulin and Phosphorus in Urine and Blood

The urine samples obtained at period-1 to period-5 were measured for their volume and then determined for the inulin and phosphorus concentrations thereof. The blood samples also obtained above were subjected to cooling centrifugation to obtain a plasma sample, which was used for determining the inulin and phosphorus concentration. The determination of inulin was conducted by Anthrone-sulfate method (Roc, L. et al., J. Biol. Chem. 178, 839-845, 1949), and the determination of phosphorus was conducted using Hitachi Automatic Analyzer 7170 model with a reagent for inorganic phosphorus determination, Autosea IP (Daiichi Pure Chemicals) in accordance with a manual (Physke-Sabaroh methods).

(4) Calculation of Inulin Clearance, Phosphorus Clearance and Fractional Excretion of Phosphorus.

Inulin clearance (Cin), phosphorus clearance (Cp) and fractional excretion of phosphorus (FEp) were calculated according to the following equations.

Calculation of Inulin Clearance (Cin):

$$Cin = \frac{U_{in} \cdot V}{P_{in}}$$

Wherein Cin represents inulin clearance (ml/kg/min); U_{in} represents the concentration of inulin in urine (mg/ml); V

US 6,903,194 B1

59

represents the amount of urine per unit time (ml/kg/min); and Pin represents the concentration of inulin in blood (mg/ml).

Calculation of phosphorus clearance (Cp):

$$Cp = Up \cdot V / Pp$$

Wherein Cp represents phosphorus clearance (ml/kg/min); Up represents the concentration of phosphorus in urine (mg/ml); V represents the amount of urine per unit time (ml/kg/min); and Pp represents the concentration of phosphorus in blood (mg/ml)

Calculation of fractional excretion of phosphorus (FEP):

$$FEP = Cp / Cin$$

Wherein FEP represents fractional excretion of phosphorus; Cin represents inulin clearance; and Cp represents phosphorus clearance. The examination was conducted using four animals.

The results were determined as the average value \pm standard error.

The results of fractional excretion of phosphorus and phosphorus concentration in blood are shown in FIGS. 25 and 26.

FIG. 25 is a graph illustrating the relationship of fractional excretion of phosphorus (=phosphorus clearance/inulin clearance) vs. periods of clearance (1 period=20 min.). The humanized anti-PtHRP antibody (1 mg/kg) was administered (i.v.) at the time of the starting of period-2.

FIG. 26 is a graph illustrating the relationship of phosphorus concentration in plasma vs. periods of clearance (1 period=20 min.). The humanized anti-PtHRP antibody (1 mg/kg) was administered (i.v.) at the time of starting of period-2.

From these results, it was found that the fractional excretion of phosphorus given after administering the antibody (i.e., from period-2 and period-5) was obviously suppressed compared with that given before administering the antibody (i.e., period-1). In other words, it was found that the administration of the neutralizing antibody to a subject developing hypophosphatemia, which causes acceleration of excretion of phosphorus (FEP>0.2) recovered the phosphorus resorption in the subject to approximately the normal level (fractional resorption of phosphate=1-FEP>0.8%) and, as a result, trend to normalcy of the phosphorus concentration in blood of the subject was observed. These results suggest the usefulness of the antibodies of the present invention as agents treating the accelerated excretion of phosphorus and hypophosphatemia caused by the presence of PtHRP.

Since PtHRP is a substrate causing malignant tumor-associated hypercalcemia, the possibility of increase in phosphorus excretion and decrease in high energy organic phosphorus in tissue caused by PtHRP is predicted. Accordingly, various diseases associated with hypophosphatemia, such as hypophosphatemic rickets and hypophosphatemic vitamin D-resistant rickets, are considered to be mainly caused by the increase in phosphorus excreted through urine and, therefore, the antibodies of the present invention would also be useful for treating these diseases.

EXAMPLE 10

Improvement of Various Clinical Symptoms of Malignant Tumor-associated Hypercalcemia

It has been known that the malignant tumor-associated hypercalcemia is caused by the presence of PtHRP which is

60

produced by the tumor and that PtHRP accelerates bone resorption and calcium resorption in the kidney and the uriniferous tubule, leading to hypercalcemia. Further, in a patient suffering from hypercalcemia, worsening of clinical symptoms, such as poor performance status, loss of consciousness, systemic malaise, hydrodipsia, nausea and emesis (anorexia) are observed. The effect of the anti-PtHRP antibody on such clinical symptoms was examined using hypercalcemia model animals of human tumor nude mouse transplantation system and human tumor-nude rat transplantation system.

As the hypercalcemia model animal, nude mice and nude rats into which human lung cancer LC-6 (purchased from the Central Institute for Experimental Animals) had been transplanted were used. Nude mice and nude rats into which human lung cancer LC-6 is transplanted tend to show increased calcium concentration in blood with increase in the tumor volume, leading hypercalcemia associated with decrease in body temperature and body weight.

The improvement effect of mouse anti-PtHRP antibody on general clinical symptoms of malignant tumor-associated hypercalcemia was examined using a human lung cancer LC-6-nude mouse transplantation system and its result is shown photographically. The effect of the antibody on improvement of decrease in spontaneous activity, body temperature and anorexia was examined using a human lung cancer LC-6-nude mouse transplantation system.

1. Improvement of Apparent Clinical Symptoms Associated With Hypercalcemia

The passage of the human lung cancer strain LC-6 was conducted using BALB/c-nu nude mice (Nippon Kurea) in vivo. For the evaluation of pharmacological efficacy, 5-weeks-old male BALB/c-nu nude mice (Nippon Kurea) were purchased and then acclimated for 1 week, and the mice of 6-weeks-old were used.

The hypercalcemia model mice were prepared and divided into groups in the following manner. The human lung cancer LC-6 was excised and then finely cut into 3 mm cube of blocks. The resultant tumor blocks were subcutaneously transplanted under the skin flap of the mice at one piece per mouse. Twenty-seven days after the transplantation, when it was confirmed that the tumor volume in each of the mice became satisfactorily large, the mice were divided into groups so that the tumor volume, the calcium concentration in blood and the body weights of the mice of the individual groups were averaged, and the mice were provided as the hypercalcemia model animals.

The tumor volume was determined by measuring the major diameter (a mm) and the minor diameter (b mm) of the tumor and calculating using the both measured values according to Galant's equation [ab²/2].

The calcium concentration in blood was determined as ionized calcium concentration in whole blood by drawing blood from each of the mice via the orbit using a hematocrit tube and applying the blood to 643 Automatic Ca⁺⁺/pH Analyzer (CIBA-CORNING).

The examination of therapeutic efficacy of the antibody on hypercalcemia was conducted in the following manner. The mouse antibody against PtHRP was administered to each of the above-mentioned hypercalcemia model animal via the tail vein on day 27, 30, 34 and 37 after transplanting the tumor in a dose amount of 100 μ g per mouse. For preparation of controls, a phosphate buffered-physiological saline was administered instead of the antibody in the same manner. On 41 days after transplanting the tumor, from each of the antibody-administered group and the control group, a typical mouse was selected and a picture was taken thereof along with a normal mouse.

US 6,903,194 B1

61

As a result, in the hypercalcemia model animal transplanted with human lung cancer LC-6, although the antibody-administered mice (shown in center of each of FIGS. 27 and 28) bore the same level of tumor mass as the control mouse (shown in the right of each of FIGS. 27 and 28), they exhibited the same level of appearance as the normal mouse (shown in the left of FIGS. 27 and 28). This result suggests that the administration of the anti-PTHrP antibody exerts an improvement in apparent-clinical symptoms (FIGS. 27 and 28).

2. Improvement of Spontaneous Activity Decrease Associated With Hypercalcemia

The passage of the human lung cancer LC-6 was conducted using BALB/c-nu/nu nude mice (Nippon Kurea) in vivo. For the evaluation of pharmacological efficacy, 5-weeks-old male F344/N Jcl-run nude rats (Nippon Kurea) were purchased and then acclimated for 1 week, and the rats of 6-weeks-old were used.

The hypercalcemia model animals were prepared in the following manner. The human lung cancer LC-6 passage was excised and then finely cut into 3 mm cube of blocks. The resultant tumor blocks were subcutaneously transplanted under the skin flap of the mice at one piece per mouse. About thirty days after the transplantation, when it was confirmed that the tumor volume in each of the mice had become satisfactorily large, the mice were divided into groups so that the tumor volume, the calcium concentration in blood and the body weights of the mice of the individual groups were averaged, and the mice were provided as the hypercalcemia model animals.

The calcium concentration in blood was determined as ionized calcium concentration in whole blood by drawing blood from each of the mice via the orbit using a hematocrit tube and applying the blood to 643 Automatic Ca^{++} pH Analyzer (CIBA-CORNING).

(1) Method for Determination Of Spontaneous Activity

The determination of spontaneous activity was conducted using ANIMEX activity meter type SE (FARAD, Electronics, Sweden), which was placed in predetermined position in the polyethylene cage in which each of the model animals was individually nurtured (watering and feeding). This apparatus was designed to measure the amount of movement of each rat. Using this apparatus, the amount of movement was recorded as the count per a definite time of period. The measurement was conducted for 13 hours (from 7:00 p.m. of a certain day to 8:00 a.m. of next day) and the results were given as count per hour.

(2) Administration of Antibody

The humanized anti-PTHrP antibody was administered each of the above-prepared rats which developed hypercalcemia via its tail vein as a control in a dose amount of 5 mg/0.5 ml/kg. Saline was administered to another group of the rats in the same manner. The measurement was conducted with alternation of an antibody-administered rat and a control rat.

The measurement was conducted on day 0 (i.e., the day before administration), 2, 4, 7 and 14 for the antibody-administered rats and on day 1, 3, 5, 8 and 15 for the control rats.

As a result, the control rats showed no change or a decrease in spontaneous activity during the test period, whereas the antibody-administered rats showed an increase in spontaneous activity on and after day 4 of the administration (FIG. 29).

3. Improvement in Body Temperature Decrease Associated With Hypercalcemia

The passage of human lung cancer LC-6 and the preparation of the malignant tumor-associated hypercalcemia model animals were conducted in the same manner as in step 2 above.

62

(1) Method for Measuring Body Temperature

The measurement of body temperature was conducted using a digital thermometer by anesthetizing the animal with pentobarbital (Nembutal, Dainippon Pharmaceutical Co., Ltd.) and inserting a temperature sensor into the rectum thereof.

(2) Administration of Antibody

The humanized anti-PTHrP antibody was administered to each of the above-mentioned hypercalcemia model rats via the tail vein in a dose amount of 1 mg/ml/kg. For a control, saline was administered to another model rat via the tail vein. Further, a normal rat to which no antibody was administered was also measured for its body temperature. The measurement of body temperature of the rats was conducted on day 0 (i.e., the day of the administration), 1, 2 and 3 after the administration with respect to all of the antibody-administered rats, the control rat and the normal rat.

As a result, the normal rat showed no change in body temperature (34.2-34.4° C) throughout the test period, whereas the malignant tumor-associated hypercalcemia model rats showed a decrease in body temperature by about 2° C. compared with the normal rats. When the humanized anti-PTHrP antibody was administered to the model rats, it was confirmed that the malignant tumor-associated hypercalcemia model rats recover their decreased body temperatures to the same level as that of the normal rats three days after administration. These results suggest that the humanized anti-PTHrP antibody of the present invention is effective for improving the body temperature decrease of the malignant tumor-associated hypercalcemia model animal (FIG. 30).

4. Improvement in Food Intake Decrease Induced

The passage of the human lung cancer LC-6 and the preparation of the hypercalcemia model animals were conducted in the same manner as described in above section 2. The model animals prepared were divided into groups so that the calcium concentration in blood and the body weights of the mice of the individual groups were averaged, and the mice were used in the experiments below.

(1) Measurement of Amount of Feed Intake

During the test period, the rats were individually placed into a metabolic cage and nurtured with water and feed. With respect to each of the rats, the amount ingested was determined as the amount (g) per 24 hours (starting from 9:00 a.m. of a certain day to 9:00 a.m. of the next day). The determination was conducted by measuring the total weight of the feedstock container both at 9:00 a.m. of the day (i.e., tare) and at 9:00 a.m. of the next day and calculating the weight difference therebetween.

(2) Administration of Antibody

The humanized anti-PTHrP antibody was administered to each of the hypercalcemia model rats (HHM rats) described above via its tail vein in a dose amount of 5 mg/0.5 ml/kg. Saline was administered to each of control group via its tail vein in the same manner. Saline was also administered to each of normal rats via tail vein in the same manner. With respect to all of the antibody-administered rats, the control rats and the normal rats, the determination of the amount of food intake was conducted on day 0 (i.e., previous day of the administration to the day of the administration), day 1 (i.e., period from the day of the administration to the next day), day 3 (i.e., period from three days after the administration to the next day) and day 5 (i.e., period from five days after the administration to the next day).

As a result, before administering the antibody, the amount ingested by the hypercalcemia model rats (5-9 rats) was 8.11

US 6,903,194 B1

63

g in average, whereas that of the normal rats was 12.06 g in average, which demonstrates an obvious decrease in the amount of ingestion by hypercalcemia model rats. When the humanized anti-PTHrP antibody was administered to the model rats, on and after the day after administering the antibody, although almost no change was observed in the amount of ingestion by the control rats, the amount of ingested by the antibody-administered rats recovered to the same level of that in the normal rats. These results suggest that the humanized anti-PTHrP antibody of the present invention is effective in improving the decrease in the amount ingested for the malignant tumor-associated hypercalcemia model (Table 6).

TABLE 6

		<u>Effect on ingestion</u>				
Individual		Eating amount of individual (g)				
Animal	No.	Administration*	day 0	day 1	day 3	day 5
Normal rat	1	Saline	13.7	16.7	18.63	18.71
	2	Saline	14.27	15.3	19.55	19.39
	3	Saline	9.83	15.5	20.72	19.88
	4	Saline	10.42	15.04	20.28	22.03
HHM rat	5	Saline	10.77	14.24	12.66	11.82
	6	Saline	6.99	8.92	2.59	14.8
HHM rat	7	Anti-PTHrP antibody	7.46	17.65	22.52	17.99
	8	Anti-PTHrP antibody	12	12.38	20.94	23.1
	9	Anti-PTHrP antibody	3.35	16.65	20.36	21.89

*Administration of saline (Saline): 0.5 ml/kg, via tail vein; and Administration of antibody: 5 mg/0.5 ml/kg, via tail vein.

From the above-mentioned results, it was demonstrated that the chimeric antibodies and the humanized antibodies of the present invention are useful as agents for improving the various clinical symptoms of malignant tumor-associated hypercalcemia.

5. Improvement of Decrease in Blood pH Induced by Hypercalcemia

The passage of human lung cancer LC-6 and the preparation of the malignant tumor-associated hypercalcemia model animals were conducted in the same manner as in step 2 above. The model animals were divided into groups so that the calcium concentration in blood and the body weight of the mice of the individual groups were averaged.

(1) Determination of Blood pH

Blood was collected from each test animal using a heparin-treated injection syringe by cardiac blood drawing

64

technique and then applying the resultant blood sample to 643 Automatic Ca²⁺/pH Analyzer (CIBA-CORNING) to determine pH of the blood sample.

(2) Administration of Antibody

The humanized anti-PTHrP antibody was administered to each of the above-mentioned hypercalcemia model rats (HHM rats) via its tail vein in a dose amount of 5 mg/0.5 ml/kg (n=3). Saline was administered to each of control group via its tail vein in the same manner (n=2). With respect to any of the antibody-administered rats and the control rats, the determination of blood pH was conducted on day 0 (i.e., the day of administration), day 1 and day 7. The results are given as average of the pH values obtained.

As a result, before administering the antibody, the pH of the blood obtained from the hypercalcemic model rats was about 7.49 (whereas that from the normal rats was 7.40±0.02), which means that the model rats obviously had developed metabolic alkalosis. When the humanized anti-PTHrP antibody of the present invention was administered to the model rats, although the control rats showed almost no change in blood pH, the antibody-administered rats showed such an improvement in pH values that the pH values recovered to near the pH value of the normal rats seven days after administering the antibody. As one of the clinical symptoms of malignant tumor-associated hypercalcemia (HHM), metabolic alkalosis has been reported which is known to be induced by the inhibition of excretion of bicarbonate ion (HCO₃⁻) in kidney. Since the administration of the humanized anti-PTHrP antibody of the present invention normalized the blood pH in the hypercalcemia model animals, it is suggested that the antibody can improve the metabolic alkalosis found in HHM (FIG. 31).

From the results mentioned above, it was demonstrated that the chimeric antibodies and the humanized antibodies of the present invention are useful as agents for improving the clinical symptoms of malignant tumor-associated hypercalcemia.

INDUSTRIAL APPLICABILITY

According to the present invention, a chimeric antibody and a humanized antibody against PTHrP are provided. These antibodies have a low antigenicity against human and therefore are useful as agents for treating hypercalcemia, hypophosphatemia and the like.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 113

<210> SEQ ID NO 1

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

DNA

<400> SEQUENCE: 1

aaatagccot tgaccagcca

20

<210> SEQ ID NO 2

<211> LENGTH: 38

<212> TYPE: DNA

US 6,903,194 B1

65

66

-continued

```

<21> ORGANISM: Artificial Sequence
<22> FEATURE:
<23> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 2

ctggttcggc ccacctctga aggtccaga atcgatag
                                     38

<210> SEQ ID NO 3
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 3

ggatcccggg ccagtgata gacagatg
                                     28

<210> SEQ ID NO 4
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 4

ggatcccggg tcagrggaag gtggraaca
                                     29

<210> SEQ ID NO 5
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 5

gttttccag tcacgac
                                     17

<210> SEQ ID NO 6
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 6

cagggaacag ctatgac
                                     17

<210> SEQ ID NO 7
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 7

gtctaagctt ccaccatgaa acttcgggct c
                                     31

<210> SEQ ID NO 8
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

```

US 6,903,194 B1

67

68

-continued

```

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 8
tggtggatcc ctgcagagac agtgaccaga                               30

<210> SEQ ID NO 9
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 9
gtctgaattc aagcttcac catgggggtt ggggtg                               36

<210> SEQ ID NO 10
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 10
tttccgggc ccttggtgga ggctgaggag acggtgacca g                               41

<210> SEQ ID NO 11
<211> LENGTH: 109
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 11
gtctgaattc aagcttagta cttggccagc ccaaggccaa cccacggtc aacctgttc       60
cgccctcttc tgaggagctc caagccaaca aggcacact agtgtgtct                               109

<210> SEQ ID NO 12
<211> LENGTH: 110
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 12
ggtttggtgg totccaatcc cgccctgacg ggggtgcoat ctgccttcca ggccactgtc       60
acagctcccg ggtagaagtc actgatcaga cacactagtg tggcctgttt                               110

<210> SEQ ID NO 13
<211> LENGTH: 98
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 13
ggagtggaga ccaccaaac ccctcaaacg agcaacaaca agtacgccc cagcagctac       60
ctgagcctga cgcccgagca gtggaagtc cacagaag                               98

```

69

US 6,903,194 B1

70

-continued

```

<210> SEQ ID NO 14
<211> LENGTH: 106
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 14
tggtgaattc ttactatgaa cattctgtag gggccactgt cttctccacg gtgctccott      60
catgcgtgac ctggcagctg tagctctgtt gggacttcca ctgctc      106

<210> SEQ ID NO 15
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 15
gtctgaattc aagcttagta cttggccage ccaaggccaa ccc      43

<210> SEQ ID NO 16
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 16
tggtgaattc ttactatgaa      20

<210> SEQ ID NO 17
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 17
caacaagtac ggcgcagca gctacctgag cctgacgc      39

<210> SEQ ID NO 18
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 18
gtagctgtg gccgcgtact tgttgtgtct ctgtttgga      39

<210> SEQ ID NO 19
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 19
gtctgaattc aagcttagtc ctaggtcgaa ctgtggctgc accatc      46

```

US 6,903,194 B1

71

72

-continued

```

<210> SEQ ID NO 20
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 20
tggtgaattc ttactaacac tctccctgt tga
34

<210> SEQ ID NO 21
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 21
gtotaagctt ccacatgga ctggactct ctct
35

<210> SEQ ID NO 22
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 22
tggtgaattc agatataact acttaacctag gacagtgaac ttggtccc
48

<210> SEQ ID NO 23
<211> LENGTH: 128
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 23
gtctaagctt ccacatggg gttgggctg agctgggttt tctcgttgc tcttttaaga
60
gggtgcacgt gtcaggtgca gctggtggag tctgggggag gcgtggtcca gctggggag
120
tccctgag
128

<210> SEQ ID NO 24
<211> LENGTH: 125
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 24
accattagta gtgtggtgag ttacacctac tatocagaca gtgtgaagg gogattcacc
60
attocagag acaattocaa gaacagctg tatotgcana tgaacagcct gagagctgag
120
gacac
125

<210> SEQ ID NO 25
<211> LENGTH: 132
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

```


US 6,903,194 B1

73

74

-continued

```

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 25
ctaccaccac tactaatggt tgcacccac tccagccctc tgcctggagc ctggcggacc      60
caagacatgc catagctact gaaggtgat ccagaggctg cacaggagag tctcagggac      120
ctcccaggct gg                                                    132

<210> SEQ ID NO 26
<211> LENGTH: 110
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 26
tgttggatcc ctgaggagac ggtgaccag gtcctctggc cccagtaagc aaagtaagtc      60
atagtagtct gtctgcaca gtaatacaca gccgtgtctt cagctctcag            110

<210> SEQ ID NO 27
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 27
gtctaaagctt ccacatggg gtttgggctg                                    30

<210> SEQ ID NO 28
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 28
tgttggatcc ctgaggagac ggtgaccag                                    30

<210> SEQ ID NO 29
<211> LENGTH: 133
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 29
acaaagcttc caccatggc tggactctc tcttctctt ctttctctt cattgtctag      60
gttctttctc ccagcttggt gtgactcaat ggcctctgct ctctgctccc ctggaggcct      120
cggtcaagct cac                                                    133

<210> SEQ ID NO 30
<211> LENGTH: 118
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

```

US 6,903,194 B1

75

76

-continued

<400> SEQUENCE: 30

agcaagatgg aagccacagc acaggtgatg ggattctga tcgtctctca ggctccagct 60

ctggggctga ggcctacctc acaatctcca gctccagtc tgaagatgag gctgacta 118

<210> SEQ ID NO 31

<211> LENGTH: 128

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 31

ctgtggcttc catcttgctt aagttccatc aagtaaccag ggccctctctc tggctgctgc 60

tgatgccatt caatgggtga cgtactgtgc tgactactca aggtgcaggt gagcttgacc 120

gaggctcc 128

<210> SEQ ID NO 32

<211> LENGTH: 114

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 32

cttggatccg ggctgaacta ggaaggctag ttgggtccct ccgcgaaca cctccacaaa 60

ttgttcctta attgtatcac ccacaccaca gtaatatgta gctctactct caga 114

<210> SEQ ID NO 33

<211> LENGTH: 17

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 33

acaaagcttc caccatg 17

<210> SEQ ID NO 34

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 34

cttggatccg ggctgaacta 19

<210> SEQ ID NO 35

<211> LENGTH: 75

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic DNA

<400> SEQUENCE: 35

cttggatccg ggctgaacta ggaaggctag ttgggtccct ccgcgaaca cgtacacaaa 60

ttgttcctta attgt 75

US 6,903,194 B1

77

78

-continued

```

<210> SEQ ID NO 36
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 36
aaagatcct taagatccat caagtaccga gggggcttct ctg                               43

<210> SEQ ID NO 37
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 37
acaaagctta gcgtacctc accatctcca gctccagcc tgagga                               46

<210> SEQ ID NO 38
<211> LENGTH: 111
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 38
cttggtatcg ggctgaacta ggaaggtcag ttgggtccct ccgcgaaca cgtacacaaa          60
ttgttcctta attgtatcac ccaaccacaa gatatactga gcttcactct c                  111

<210> SEQ ID NO 39
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 39
ctctctctgc tgctgtgat accattcaat ggtgtacgta ct                               42

<210> SEQ ID NO 40
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 40
cgaggggccct tctctggctg ctgctg                               26

<210> SEQ ID NO 41
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA

<400> SEQUENCE: 41
gagaagggcc ctargtacct gatgrawett aagca                               35

```

79

US 6,903,194 B1

80

-continued

```

<210> SEQ ID NO 42
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 42
cacgaattca ctatgatctc tggaaacctc agagg                               35

<210> SEQ ID NO 43
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 43
ggcttggagc toctcaga                                                  18

<210> SEQ ID NO 44
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      DNA
<400> SEQUENCE: 44
gacagtggtt caaagttttt                                              20

<210> SEQ ID NO 45
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 45
Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser Leu Gly Ala
 1             5             10             15
Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
      20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys Tyr Val Met
      35             40             45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
      50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu Ser Ile Ser
      65             70             75             80
Asn Ile Gln Pro Glu Asp Gln Ala Met Tyr Ile Cys Gly Val Gly Asp
      85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Val
      100            105            110
Thr Val Leu Gly Gln Pro
      115

<210> SEQ ID NO 46
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 46

```

US 6,903,194 B1

81

82

-continued

Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys Pro Gly Gly
 1 5 10 15
 Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
 20 25 30
 Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp Val
 35 40 45
 Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met Phe Tyr Cys
 85 90 95
 Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110
 Leu Val Thr Val Ser Ala
 115

<210> SEQ ID NO 47

<211> LENGTH: 116

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 47

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 35 40 45
 Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly
 115

<210> SEQ ID NO 48

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 48

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80

US 6,903,194 B1

83

84

-continued

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 49

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 49

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 50

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 50

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 51

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

US 6,903,194 B1

85

86

-continued

<400> SEQUENCE: 51

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
 35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
 85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
 100 105 110

Thr Val Leu Gly Gln Pro
 115

<210> SEQ ID NO 52

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 52

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
 35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
 100 105 110

Thr Val Leu Gly Gln Pro
 115

<210> SEQ ID NO 53

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 53

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60

US 6,903,194 B1

87

88

-continued

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 54

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 54

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 55

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 55

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 56

<211> LENGTH: 118

US 6,903,194 B1

89

90

-continued

```

<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 56
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1             5             10             15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
                20             25             30
Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35             40             45
Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
 50             55             60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65             70             75             80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85             90             95
Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
100             105             110
Leu Val Thr Val Ser Ser
115

<210> SEQ ID NO 57
<211> LENGTH: 411
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURES:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(411)
<221> NAME/KEY: sig.peptide
<222> LOCATION: (1)..(57)
<221> NAME/KEY: mat.peptide
<222> LOCATION: (58)..(411)

<400> SEQUENCE: 57
atg aac ttc ggg ctc agc ttg att ttc ctt gcc ctc att tta aaa ggt      48
Met Asn Phe Gly Leu Ser Leu Ile Phe Leu Ala Leu Ile Leu Lys Gly
                -15             -10             -5
gtc cag tgt gag gtg caa ctg gtg gag tct ggg gga gac tta gtg aag      96
Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys
-1 1             5             10
cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc      144
Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
15             20             25
agt agc tat ggc atg tct tgg att cgc cag act cca gac aag agg ctg      192
Ser Ser Tyr Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu
30             35             40             45
gag tgg gtc gca acc att agt agt ggt ggt agt tac acc tac tat cca      240
Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
50             55             60
gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat gcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
65             70             75
acc cta tac ctg caa atg agc agt ctg aag tct gag gac aca gcc atg      336
Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met
80             85             90
ttt tac tgt gca aga cag act act atg act tac ttt gct tac tgg ggc      384
Phe Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly
95             100             105
caa ggg act cgt gtc act gtc tct goa                                411
Gln Gly Thr Leu Val Thr Val Ser Ala
110             115

```

US 6,903,194 B1

91

92

-continued

```

<210> SEQ ID NO 58
<211> LENGTH: 411
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(411)
<221> NAME/KEY: sig.peptide
<222> LOCATION: (1)..(57)
<221> NAME/KEY: mat.peptide
<222> LOCATION: (58)..(411)

<400> SEQUENCE: 58

atg ggg ttt ggg ctg agc tgg gtt ttc ctc gtt gct ctt tta aga ggt      48
Met Gly Phe Gly Leu Ser Trp Val Phe Leu Val Ala Leu Leu Arg Gly
      -15                      -10                      -5

gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc gtg gtc cag      96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Val Val Gln
      -1      1                      5                      10

cct ggg agt tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc      144
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
      15                      20                      25

agt agc tat ggc atg tct tgg gtc cgc cag gct oca ggc aag ggg ctg      192
Ser Ser Tyr Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
      30                      35                      40                      45

gag tgg gtg gca aac att agt agt ggt ggt agt tac aac tac tat oca      240
Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Pro
      50                      55                      60

gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat tcc aag aac      288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
      65                      70                      75

acg ctg tat ctg oca atg aac agc ctg aga gct gac aac gct gtg      336
Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
      80                      85                      90

tat tac tgt ggc aga cag act act atg act tac ttt gct tsc tgg ggc      384
Tyr Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly
      95                      100                      105

cag gga acc ctg gtc acc gtc tcc tca      411
Gln Gly Thr Leu Val Thr Val Ser Ser
      110                      115

<210> SEQ ID NO 59
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 59

Lys Ala Ser Gln Asp Val Asn Thr Ala Val Ala
      1                      5                      10

<210> SEQ ID NO 60
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 60

Ser Ala Ser Asn Arg Tyr Thr
      1                      5

<210> SEQ ID NO 61
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

```

US 6,903,194 B1

93

94

-continued

<400> SEQUENCE: 61

Gln Gln His Tyr Ser Thr Pro Phe Thr
 1 5

<210> SEQ ID NO 62

<211> LENGTH: 5

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 62

Pro Tyr Trp Met Gln
 1 5

<210> SEQ ID NO 63

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 63

Ser Ile Phe Gly Asp Gly Asp Thr Arg Tyr Ser Gln Lys Phe Lys Gly
 1 5 10 15

<210> SEQ ID NO 64

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 64

Gly Leu Arg Arg Gly Gly Tyr Tyr Phe Asp Tyr
 1 5 10

<210> SEQ ID NO 65

<211> LENGTH: 411

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (1)..(411)

<221> NAME/KEY: sig.peptide

<222> LOCATION: (1)..(57)

<221> NAME/KEY: mat.peptide

<222> LOCATION: (58)..(411)

<400> SEQUENCE: 65

atg gcc tgg act cct ctc ttc ttc ttt gtt ctt cat tgc tca ggt 48
 Met Ala Trp Thr Pro Leu Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5

tat ttc tcc caa ott gtg ctc act cag tca tct tca gcc tct ttc tcc 96
 Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser
 -1 1 5 10

ctg gga gcc tca gca aaa ctc acg tgc acc ttg agt agt cag cac agt 144
 Leu Gly Ala Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25

acg tac acc att gaa tgg tat cag caa cag cca ctc aag cct cct aag 192
 Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys
 30 35 40 45

tat gtg gat gat ctt aag caa gat gga agc cag agc aca ggt gat ggg 240
 Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60

att cct gat cgc ttc tct gga tcc agc tct ggt gct gat cgc tac ctt 288
 Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu
 65 70 75

agc att tcc aac atc cag cca gaa gat gaa gca atg tac atc tgt ggt 336

US 6,903,194 B1

95

96

-continued

Ser Ile Ser Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly	
80 85 90	
gtg ggt gat aca att aag gaa caa ttt gtg tat gtt ttc ggc ggt ggg	384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly	
95 100 105	
acc aag gtc act gtc cta ggt cag ccc	411
Thr Lys Val Thr Val Leu Gly Gln Pro	
110 115	
<210> SEQ ID NO 66	
<211> LENGTH: 405	
<212> TYPE: DNA	
<213> ORGANISM: Homo sapiens	
<220> FEATURE:	
<221> NAME/KEY: CDS	
<222> LOCATION: (1)..(405)	
<221> NAME/KEY: sig.peptide	
<222> LOCATION: (1)..(57)	
<221> NAME/KEY: mat.peptide	
<222> LOCATION: (58)..(405)	
<400> SEQUENCE: 66	
atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt	48
Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly	
-15 -10 -5	
tat ttc tcc gac ctt gtg ctg act caa tgg ccc tct gcc tct gcc tcc	96
Ser Phe Ser Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser	
-1 1 5 10	
ctg gga gcc tgg gtc aag ctc acc tgc acc ttg agt agt cag cac agt	144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser	
15 20 25	
acg tac acc att gaa tgg cat cag cag cag oca gag aag ggc cct cgg	192
Thr Tyr Thr Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg	
30 35 40 45	
tac ttg gat aaa ctt aag caa gat gga agc cac agc aca ggt gat ggg	240
Tyr Leu Met Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly	
50 55 60	
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc	288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu	
65 70 75	
acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt	336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly	
80 85 90	
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg	384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly	
95 100 105	
acc aaa ctg acc gtc cta ggt	405
Thr Lys Leu Thr Val Leu Gly	
110 115	
<210> SEQ ID NO 67	
<211> LENGTH: 411	
<212> TYPE: DNA	
<213> ORGANISM: Homo sapiens	
<220> FEATURE:	
<221> NAME/KEY: CDS	
<222> LOCATION: (1)..(411)	
<221> NAME/KEY: sig.peptide	
<222> LOCATION: (1)..(57)	
<221> NAME/KEY: mat.peptide	
<222> LOCATION: (58)..(411)	
<400> SEQUENCE: 67	
atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt	48
Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly	

US 6,903,194 B1

97

98

-continued

	-15	-10	-5	
tct ttc tcc cag ctt gtg ctg act caa tgg ccc tct gcc tct gcc tcc				96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser				
-1 1 5 10				
ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt				144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser				
15 20 25				
acg tac acc att gaa tgg tat cag cag cag oca gag aag ggc oct aag				192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys				
30 35 40 45				
tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg				240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly				
50 55 60				
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc				288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu				
65 70 75				
acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt				336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly				
80 85 90				
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc gcc gga ggg				384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly				
95 100 105				
acc aaa ctg acc gtc cta ggc cag ccc				411
Thr Lys Leu Thr Val Leu Gly Gln Pro				
110 115				
<210> SEQ ID NO 68				
<211> LENGTH: 411				
<212> TYPE: DNA				
<213> ORGANISM: Homo sapiens				
<220> FEATURES:				
<221> NAME/KEY: CDS				
<222> LOCATION: (1)..(411)				
<221> NAME/KEY: sig.peptide				
<222> LOCATION: (1)..(57)				
<221> NAME/KEY: mat.peptide				
<222> LOCATION: (58)..(411)				
<400> SEQUENCE: 68				
atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt				48
Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly				
-15 -10 -5				
tct ttc tcc cag ctt gtg ctg act caa tgg ccc tct gcc tct gcc tcc				96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser				
-1 1 5 10				
ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt				144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser				
15 20 25				
acg tac acc att gaa tgg tat cag cag cag oca gag aag ggc oct aag				192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys				
30 35 40 45				
tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg				240
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly				
50 55 60				
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc				288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu				
65 70 75				
acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt				336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly				
80 85 90				
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc gcc gga ggg				384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly				

US 6,903,194 B1

99

100

-continued

95	100	105	
acc aaa ctg acc gtc cta ggc cag ccc			411
Thr Lys Leu Thr Val Leu Gly Gln Pro			
110	115		
<210> SEQ ID NO 69 <211> LENGTH: 411 <212> TYPE: DNA <213> ORGANISM: Homo sapiens <220> FEATURES: <221> NAME/KEY: CDS <222> LOCATION: (1)..(411) <221> NAME/KEY: sig.peptide <222> LOCATION: (1)..(57) <221> NAME/KEY: mat.peptide <222> LOCATION: (58)..(411)			
<400> SEQUENCE: 69			
atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt			48
Met Ala Thr Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly			
	-15	-10	-5
tot ttc tcc cag ctt gtg ctg act caa tgc ccc tct gcc tct gcc tcc			96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser			
	-1 1	5	10
ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag cac agt			144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser			
	15	20	25
acg tac acc att gaa tgg tat cag cag cag cca gag aag gcc cct agg			192
Thr Tyr Thr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg			
	30	35	40
tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg			240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly			
	50	55	60
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc			288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu			
	65	70	75
acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt			336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly			
	80	85	90
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc gcc gga ggg			384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly			
	95	100	105
acc aaa ctg acc gtc cta ggc cag ccc			411
Thr Lys Leu Thr Val Leu Gly Gln Pro			
110	115		
<210> SEQ ID NO 70 <211> LENGTH: 411 <212> TYPE: DNA <213> ORGANISM: Homo sapiens <220> FEATURES: <221> NAME/KEY: CDS <222> LOCATION: (1)..(411) <221> NAME/KEY: sig.peptide <222> LOCATION: (1)..(57) <221> NAME/KEY: mat.peptide <222> LOCATION: (58)..(411)			
<400> SEQUENCE: 70			
atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt			48
Met Ala Thr Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly			
	-15	-10	-5
tot ttc tcc cag ctt gtg ctg act caa tgc ccc tct gcc tct gcc tcc			96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser			
	-1 1	5	10

US 6,903,194 B1

101

102

-continued

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt	144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser	
15 20 25	
acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg	192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg	
30 35 40 45	
tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg	240
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly	
50 55 60	
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc	288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu	
65 70 75	
acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt	336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly	
80 85 90	
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg	384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly	
95 100 105	
acc aaa ctg acc gtc cta ggc cag ccc	411
Thr Lys Leu Thr Val Leu Gly Gln Pro	
110 115	
<210> SEQ ID NO 71	
<211> LENGTH: 411	
<212> TYPE: DNA	
<213> ORGANISM: Homo sapiens	
<220> FEATURES:	
<221> NAME/KEY: CDS	
<222> LOCATION: (1)..(411)	
<221> NAME/KEY: sig.peptide	
<222> LOCATION: (1)..(57)	
<221> NAME/KEY: mat.peptide	
<222> LOCATION: (58)..(411)	
<400> SEQUENCE: 71	
atg gcc tgg act cct ctc ttc ttc ttt gtt ctt cat tgc tca ggt	48
Met Ala Trp Thr Pro Leu Phe Phe Phe Val Leu His Cys Ser Gly	
-15 -10 -5	
tct ttc tcc cag ctt gtg ctg act caa tgg ccc tct gcc tot gcc tcc	96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser	
-1 1 5 10	
ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt	144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser	
15 20 25	
acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag	192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys	
30 35 40 45	
tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg	240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly	
50 55 60	
att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc	288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu	
65 70 75	
acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt	336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly	
80 85 90	
gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg	384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly	
95 100 105	
acc aaa ctg acc gtc cta ggc cag ccc	411
Thr Lys Leu Thr Val Leu Gly Gln Pro	
110 115	

US 6,903,194 B1

103

104

-continued

```

<210> SEQ ID NO 72
<211> LENGTH: 411
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(411)
<221> NAME/KEY: sig.peptide
<222> LOCATION: (1)..(57)
<221> NAME/KEY: mat.peptide
<222> LOCATION: (58)..(411)

<400> SEQUENCE: 72

atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt      48
Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
      -15                      -10                      -5

tct ttc tcc cag ctt gtg ctg act caa tgc ccc cct gcc tct gcc tcc      96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
      -1 1                      5                      10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt      144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
      15                      20                      25

acg tac acc att gaa tgg tat cag cag cag caa gag aag ggc cct agg      192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
      30                      35                      40                      45

tac ctg atg gat ctt aag caa gat gga ago cag ago aca ggt gat ggg      240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
      50                      55                      60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc      288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
      65                      70                      75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt      336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
      80                      85                      90

gtg ggt gat acd att aag gaa caa ttt gtg tac gtg ttc gcc gga ggg      384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
      95                      100                      105

acc aaa ctg acc gtc cta ggc cag ccc      411
Thr Lys Leu Thr Val Leu Gly Gln Pro
      110                      115

```

```

<210> SEQ ID NO 73
<211> LENGTH: 411
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(411)
<221> NAME/KEY: sig.peptide
<222> LOCATION: (1)..(57)
<221> NAME/KEY: mat.peptide
<222> LOCATION: (58)..(411)

<400> SEQUENCE: 73

atg gcc tgg act cct ctc ttc ttc ttc ttt gtt ctt cat tgc tca ggt      48
Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
      -15                      -10                      -5

tct ttc tcc cag ctt gtg ctg act caa tgc ccc cct gcc tct gcc tcc      96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
      -1 1                      5                      10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt      144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
      15                      20                      25

```


US 6,903,194 B1

105

106

-continued

```

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag      192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
30          35          40          45

tac gtg atg gat ctt aag caa gat gga agc oac agc aca ggt gat ggg      240
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50          55          60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc      288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65          70          75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt      336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
80          85          90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg      384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95          100          105

acc aaa ctg acc gtc cta ggc cag ccc      411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110          115

<210> SEQ ID NO 74
<211> LENGTH: 411
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(411)
<221> NAME/KEY: sig.peptide
<222> LOCATION: (1)..(57)
<221> NAME/KEY: mat.peptide
<222> LOCATION: (58)..(411)

<400> SEQUENCE: 74

atg gcc tgg act cct ctc ttc ttc ttt gtt ctt cat tgc tca ggt      48
Met Ala Trp Thr Pro Leu Phe Phe Phe Val Leu His Cys Ser Gly
-15          -10          -5

tct ttc tcc cag ctt gtg ctg act caa tcg ccc tct gcc tct gcc tcc      96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1          1          5          10

ctg gga gcc tgc gtc aag ctc acc tgc acc ttg agt agt cag oac agt      144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15          20          25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg      192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
30          35          40          45

tac gtg atg gat ctt aag caa gat gga agc oac agc aca ggt gat ggg      240
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50          55          60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc      288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65          70          75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt      336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
80          85          90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg      384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95          100          105

acc aaa ctg acc gtc cta ggc cag ccc      411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110          115

```

```

<210> SEQ ID NO 75
<211> LENGTH: 34
<212> TYPE: PRT

```

US 6,903,194 B1

107

108

-continued

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 75

Ala	Val	Ser	Glu	His	Gln	Leu	Leu	His	Asp	Lys	Gly	Lys	Ser	Ile	Gln
1				5					10					15	
Asp	Leu	Arg	Arg	Arg	Phe	Phe	Leu	His	His	Leu	Ile	Ala	Glu	Ile	His
		20						25					30		

Thr Ala

<210> SEQ ID NO 76

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 76

Met	Asn	Phe	Gly	Leu	Ser	Leu	Ile	Phe	Leu	Ala	Leu	Ile	Leu	Lys	Gly
			-15					-10						-5	

Val	Gln	Cys	Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Asp	Leu	Val	Lys
-1	1					5					10				

Pro	Gly	Gly	Ser	Leu	Lys	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
15				20						25					

Ser	Ser	Tyr	Gly	Met	Ser	Trp	Ile	Arg	Gln	Thr	Pro	Asp	Lys	Arg	Leu
30				35						40				45	

Glu	Trp	Val	Ala	Thr	Ile	Ser	Ser	Gly	Gly	Ser	Tyr	Thr	Tyr	Tyr	Pro
		50						55						60	

Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn
		65						70					75		

Thr	Leu	Tyr	Leu	Gln	Met	Ser	Ser	Leu	Lys	Ser	Glu	Asp	Thr	Ala	Met
		80						85				90			

Phe	Tyr	Cys	Ala	Arg	Gln	Thr	Thr	Met	Thr	Tyr	Phe	Ala	Tyr	Trp	Gly
	95				100						105				

Gln	Gly	Thr	Leu	Val	Thr	Val	Ser	Ala
110				115				

<210> SEQ ID NO 77

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 77

Met	Gly	Phe	Gly	Leu	Ser	Trp	Val	Phe	Leu	Val	Ala	Leu	Leu	Arg	Gly
				-15				-10						-5	

Val	Gln	Cys	Gln	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Val	Val	Gln
-1	1					5						10			

Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
15				20						25					

Ser	Ser	Tyr	Gly	Met	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
30				35						40				45	

Glu	Trp	Val	Ala	Thr	Ile	Ser	Ser	Gly	Gly	Ser	Tyr	Thr	Tyr	Tyr	Pro
		50						55						60	

Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser	Lys	Asn
		65						70				75			

Thr	Leu	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val
		80						85				90			

Tyr	Tyr	Cys	Ala	Arg	Gln	Thr	Thr	Met	Thr	Tyr	Phe	Ala	Tyr	Trp	Gly
	95				100						105				

US 6,903,194 B1

109

110

-continued

Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

<210> SEQ ID NO 78

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 78

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
-15 -10 -5

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser
-1 1 5 10

Leu Gly Ala Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys
30 35 40 45

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu
65 70 75

Ser Ile Ser Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly
80 85 90

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

Thr Lys Val Thr Val Leu Gly Gln Pro
110 115

<210> SEQ ID NO 79

<211> LENGTH: 135

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 79

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
-15 -10 -5

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

Thr Tyr Thr Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg
30 35 40 45

Tyr Leu Met Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65 70 75

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
80 85 90

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

Thr Lys Leu Thr Val Leu Gly
110 115

<210> SEQ ID NO 80

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

US 6,903,194 B1

111

112

-continued

<400> SEQUENCE: 80

```

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
      -15                      -10                      -5
Ser Phe Ser Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
      -1  1                      5                      10
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
      15                      20                      25
Thr Tyr Thr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
      30                      35                      40                      45
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
      50                      55                      60
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
      65                      70                      75
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
      80                      85                      90
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
      95                      100                      105
Thr Lys Leu Thr Val Leu Gly Gln Pro
      110                      115

```

<210> SEQ ID NO 81

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 81

```

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
      -15                      -10                      -5
Ser Phe Ser Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
      -1  1                      5                      10
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
      15                      20                      25
Thr Tyr Thr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
      30                      35                      40                      45
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
      50                      55                      60
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
      65                      70                      75
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
      80                      85                      90
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
      95                      100                      105
Thr Lys Leu Thr Val Leu Gly Gln Pro
      110                      115

```

<210> SEQ ID NO 82

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 82

```

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
      -15                      -10                      -5
Ser Phe Ser Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
      -1  1                      5                      10
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

```

US 6,903,194 B1

113

114

-continued

15	20	25
Thr Tyr Thr Ile Glu Trp Tyr	Gln Gln Gln Pro	Glu Lys Gly Pro Arg
30	35	40 45
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly		
	50	55 60
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu		
	65	70 75
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly		
	80	85 90
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly		
	95	100 105
Thr Lys Leu Thr Val Leu Gly Gln Pro		
110	115	

<210> SEQ ID NO 83

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 83

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly		
	-15	-10 -5
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser		
	-1 1	5 10
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser		
	15	20 25
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg		
	30	35 40 45
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly		
	50	55 60
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu		
	65	70 75
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly		
	80	85 90
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly		
	95	100 105
Thr Lys Leu Thr Val Leu Gly Gln Pro		
110	115	

<210> SEQ ID NO 84

<211> LENGTH: 137

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 84

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly		
	-15	-10 -5
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser		
	-1 1	5 10
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser		
	15	20 25
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys		
	30	35 40 45
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly		
	50	55 60
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu		

US 6,903,194 B1

115

116

-continued

65					70					75					
Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Glu	Ala	Asp	Tyr	Ile	Cys	Gly
				80					85						90
Val	Gly	Asp	Thr	Ile	Lys	Glu	Gln	Phe	Val	Tyr	Val	Phe	Gly	Gly	Gly
				95					100						105
Thr	Lys	Leu	Thr	Val	Leu	Gly	Gln	Pro							
				110					115						

<210> SEQ ID NO 85
 <211> LENGTH: 137
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 85

Met	Ala	Trp	Thr	Pro	Leu	Phe	Phe	Phe	Phe	Val	Leu	His	Cys	Ser	Gly
				-15					-10						-5
Ser	Phe	Ser	Gln	Leu	Val	Leu	Thr	Gln	Ser	Pro	Ser	Ala	Ser	Ala	Ser
			-1	1				5						10	
Leu	Gly	Ala	Ser	Val	Lys	Leu	Thr	Cys	Thr	Leu	Ser	Ser	Gln	His	Ser
			15				20						25		
Thr	Tyr	Thr	Ile	Glu	Trp	Tyr	Gln	Gln	Gln	Pro	Glu	Lys	Gly	Pro	Arg
			30				35						40		45
Tyr	Leu	Met	Asp	Leu	Lys	Gln	Asp	Gly	Ser	His	Ser	Thr	Gly	Asp	Gly
				50				55						60	
Ile	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Ser	Ser	Gly	Ala	Glu	Arg	Tyr	Leu
				65				70						75	
Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Glu	Ala	Asp	Tyr	Ile	Cys	Gly
				80				85						90	
Val	Gly	Asp	Thr	Ile	Lys	Glu	Gln	Phe	Val	Tyr	Val	Phe	Gly	Gly	Gly
				95				100						105	
Thr	Lys	Leu	Thr	Val	Leu	Gly	Gln	Pro							
				110				115							

<210> SEQ ID NO 86
 <211> LENGTH: 137
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <400> SEQUENCE: 86

Met	Ala	Trp	Thr	Pro	Leu	Phe	Phe	Phe	Phe	Val	Leu	His	Cys	Ser	Gly
				-15					-10						-5
Ser	Phe	Ser	Gln	Leu	Val	Leu	Thr	Gln	Ser	Pro	Ser	Ala	Ser	Ala	Ser
			-1	1				5						10	
Leu	Gly	Ala	Ser	Val	Lys	Leu	Thr	Cys	Thr	Leu	Ser	Ser	Gln	His	Ser
			15				20						25		
Thr	Tyr	Thr	Ile	Glu	Trp	Tyr	Gln	Gln	Gln	Pro	Glu	Lys	Gly	Pro	Lys
			30				35						40		45
Tyr	Val	Met	Asp	Leu	Lys	Gln	Asp	Gly	Ser	His	Ser	Thr	Gly	Asp	Gly
				50				55						60	
Ile	Pro	Asp	Arg	Phe	Ser	Gly	Ser	Ser	Ser	Gly	Ala	Glu	Arg	Tyr	Leu
				65				70						75	
Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Glu	Ala	Asp	Tyr	Ile	Cys	Gly
				80				85						90	
Val	Gly	Asp	Thr	Ile	Lys	Glu	Gln	Phe	Val	Tyr	Val	Phe	Gly	Gly	Gly
				95				100						105	
Thr	Lys	Leu	Thr	Val	Leu	Gly	Gln	Pro							

US 6,903,194 B1

117

118

-continued

110 115

<210> SEQ ID NO 87
 <211> LENGTH: 137
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 87

Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly
 -15 -10 -5

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
 -1 1 5 10

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
 15 20 25

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
 30 35 40 45

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
 50 55 60

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
 65 70 75

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
 80 85 90

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
 95 100 105

Thr Lys Leu Thr Val Leu Gly Gln Pro
 110 115

<210> SEQ ID NO 88
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Murine sp.

<400> SEQUENCE: 88

Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys Pro Gly Gly
 1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
 20 25 30

Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu Glu Trp Val
 35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80

Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met Phe Tyr Cys
 85 90 95

Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
 100 105 110

Leu Val Thr Val Ser Ala
 115

<210> SEQ ID NO 89
 <211> LENGTH: 115
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 89

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15

US 6,903,194 B1

119

120

-continued

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Val Ile Ser Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Glu Ser Arg Gly Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
100 105 110

Val Ser Ser
115

<210> SEQ ID NO 90
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
peptide

<400> SEQUENCE: 90

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr
100 105 110

Leu Val Thr Val Ser Ser
115

<210> SEQ ID NO 91
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Murine sp.

<400> SEQUENCE: 91

Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser Leu Gly Ala
1 5 10 15

Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu Ser Ile Ser
65 70 75 80

US 6,903,194 B1

121

122

-continued

Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Val
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 92

<211> LENGTH: 99

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 92

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gly His Ser Ser Tyr Ala
20 25 30

Ile Ala Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
35 40 45

Lys Leu Asn Ser Asp Gly Ser His Ser Lys Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gln Thr Trp Gly
85 90 95

Thr Gly Ile

<210> SEQ ID NO 93

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 93

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
35 40 45

Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 94

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

123

US 6,903,194 B1

124

-continued

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 94

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
35 40 45

Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 95

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 95

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp His Gln Gln Gln Pro Glu Lys Pro Pro Arg Tyr Leu Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 96

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 96

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr

US 6,903,194 B1

125

126

-continued

20	25	30
Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met		
35	40	45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp		
50	55	60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser		
65	70	75
Ser Leu Gln Pro Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp		
85	90	95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu		
100	105	110
Thr Val Leu Gly Gln Pro		
115		

<210> SEQ ID NO 97
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 97

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala		
1	5	10
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr		
20	25	30
Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met		
35	40	45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp		
50	55	60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser		
65	70	75
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp		
85	90	95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu		
100	105	110
Thr Val Leu Gly Gln Pro		
115		

<210> SEQ ID NO 98
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 98

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala		
1	5	10
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr		
20	25	30
Ile Glu Trp His Gln Gln Gln Pro Glu Lys Pro Pro Arg Tyr Leu Met		
35	40	45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp		
50	55	60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser		

US 6,903,194 B1

127

128

-continued

65	70	75	80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp			
	85	90	95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu			
	100	105	110
Thr Val Leu Gly Gln Pro			
	115		
<210> SEQ ID NO 99			
<211> LENGTH: 118			
<212> TYPE: PRT			
<213> ORGANISM: Artificial Sequence			
<220> FEATURES:			
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide			
<400> SEQUENCE: 99			
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala			
1	5	10	15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr			
	20	25	30
Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met			
	35	40	45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp			
	50	55	60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser			
	65	70	75
Ser Leu Gln Pro Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp			
	85	90	95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu			
	100	105	110
Thr Val Leu Gly Gln Pro			
	115		
<210> SEQ ID NO 100			
<211> LENGTH: 118			
<212> TYPE: PRT			
<213> ORGANISM: Artificial Sequence			
<220> FEATURES:			
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide			
<400> SEQUENCE: 100			
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala			
1	5	10	15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr			
	20	25	30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met			
	35	40	45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp			
	50	55	60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser			
	65	70	75
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp			
	85	90	95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu			
	100	105	110
Thr Val Leu Gly Gln Pro			

129

US 6,903,194 B1

130

-continued

115

<210> SEQ ID NO 101
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 101

```

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
                20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
                35             40             45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
                50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
                65             70             75             80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
                85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
                100            105            110
Thr Val Leu Gly Gln Pro
                115

```

<210> SEQ ID NO 102
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 102

```

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
                20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
                35             40             45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
                50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
                65             70             75             80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
                85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
                100            105            110
Thr Val Leu Gly Gln Pro
                115

```

<210> SEQ ID NO 103
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic

131

US 6,903,194 B1

132

-continued

```

peptide

<400> SEQUENCE: 103
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
          20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
          35             40             45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
          50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
          65             70             75             80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
          85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
          100             105             110
Thr Val Leu Gly Gln Pro
          115

<210> SEQ ID NO 104
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
      peptide

<400> SEQUENCE: 104
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
          20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
          35             40             45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
          50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
          65             70             75             80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
          85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
          100             105             110
Thr Val Leu Gly Gln Pro
          115

<210> SEQ ID NO 105
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
      peptide

<400> SEQUENCE: 105
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
          20             25             30

```

133

US 6,903,194 B1

134

-continued

```

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
   35                     40                     45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
   50                     55                     60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
   65                     70                     75                     80
Ser Leu Gln Ser Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
                      85                     90                     95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
   100                     105                     110
Thr Val Leu Gly Gln Pro
   115

<210> SEQ ID NO 106
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
      peptide

<400> SEQUENCE: 106
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
  1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
      20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
   35                     40                     45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
   50                     55                     60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
   65                     70                     75                     80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
                      85                     90                     95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
   100                     105                     110
Thr Val Leu Gly Gln Pro
   115

```

```

<210> SEQ ID NO 107
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic
      peptide

<400> SEQUENCE: 107
Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
  1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
      20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
   35                     40                     45
Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
   50                     55                     60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
   65                     70                     75                     80

```

US 6,903,194 B1

135

136

-continued

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 108

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 108

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> SEQ ID NO 109

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 109

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
35 40 45

Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

US 6,903,194 B1

137

138

-continued

<210> SEQ ID NO 110
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 110

```

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
                20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Leu Met
 35             40             45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65             70             75
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100            105            110
Thr Val Leu Gly Gln Pro
115

```

<210> SEQ ID NO 111
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 111

```

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1             5             10             15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
                20             25             30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Leu Met
 35             40             45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50             55             60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65             70             75
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85             90             95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100            105            110
Thr Val Leu Gly Gln Pro
115

```

<210> SEQ ID NO 112
 <211> LENGTH: 118
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

US 6,903,194 B1

139

140

-continued

<400> SEQUENCE: 112

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Gln Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly Gln Pro
 115

<210> SEQ ID NO 113

<211> LENGTH: 118

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide

<400> SEQUENCE: 113

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
 1 5 10 15
 Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
 20 25 30
 Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
 35 40 45
 Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
 50 55 60
 Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
 65 70 75 80
 Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
 85 90 95
 Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Thr Lys Leu
 100 105 110
 Thr Val Leu Gly Gln Pro
 115

What is claimed is:

1. An isolated polypeptide comprising an L chain V region of a humanized antibody comprising an amino acid sequence chosen from SEQ ID NOs: 48-51.

2. An isolated polypeptide comprising an L chain V region of a humanized antibody comprising an amino acid sequence chosen from SEQ ID NOs: 52-55.

3. An isolated polypeptide comprising an H chain V region of a humanized antibody comprising an amino acid sequence as shown in SEQ ID NO: 56.

4. An isolated L chain of a humanized antibody which binds to a human parathyroid hormone related protein, comprising a polypeptide comprising an L chain C region of

55 a human antibody and a polypeptide comprising an L chain V region according to claim 1 or 2.

5. An isolated H chain of a humanized antibody which binds to a human parathyroid hormone related protein, comprising a polypeptide comprising an H chain C region of a human antibody and a polypeptide comprising an H chain V region according to claim 3.

6. An isolated humanized antibody comprising an L chain of a humanized antibody according to claim 4 and an H chain of a humanized antibody according to claim 5, wherein the humanized antibody binds to a human parathyroid hormone related protein.

7. An agent for suppressing hypercalcemia comprising the humanized antibody of claim 6 as an effective ingredient.

US 6,903,194 B1

141

8. An agent for suppressing hypercalcemia associated with malignant tumor, comprising the humanized antibody of claim 6 as an effective ingredient.

9. The agent for suppressing hypercalcemia according to claim 8, wherein the malignant tumor is at least one chosen from cancer of the pancreas, lung cancer, cancer of the pharynx, cancer of the larynx, cancer of the tongue, cancer of the gingiva, cancer of the esophagus, cancer of the stomach, cancer of the biliary duct, cancer of the breast, cancer of the kidney, cancer of the urinary bladder, cancer of the uterus and prostate cancer, and malignant lymphoma.

10. An agent for improving hypophosphatemia comprising, as an active ingredient, a substance capable of

142

inhibiting the binding between parathyroid hormone related protein (PTHrP) and a receptor thereof,

wherein the substance is the humanized antibody of claim 6.

11. A method of inhibiting the binding between PTHrP and a receptor thereof, comprising:

combining the humanized antibody of claim 6 with the PTHrP;

allowing said humanized antibody to bind to PTHrP; and inhibiting the binding of PTHrP to a PTHrP receptor.

* * * * *

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

MASSFELDER et al.Atty. Ref.: **3665-133**Serial No. **10/520,085**Group: **1643**Filed: **January 5, 2005**Examiner: **Gussow**For: **USE OF PTHRP ANTAGONISTS FOR TREATING RENAL
CELL CARCINOMA**

* * * * *

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

RULE 132 DECLARATION

I, Thierry MASSFELDER, do hereby declare and say as follows:

1. I am aware of the above-identified application, and am fully informed of the invention claimed therein. I am inventor of the subject matter claimed in the above.

3. I am currently a Researcher (tenure) in INSERM (French Institute of Health and Medical Research), Team leader "search of new therapeutic and pronostic options for human kidney cancer", Renal Pharmacology and Physiopathology Laboratory INSERM Unit 727, Université Louis Pasteur, 11, rue Humann, Bât 4, 1er étage, 67085 Strasbourg Cedex. I hold an Extensive Research Degree ("D.E.A" in French) in Pharmacology and Pharmacochimistry, Molecular and Cellular Pharmacology option, from Louis Pasteur University (ULP), Strasbourg, FRANCE, earned in 1991; obtained with distinction (mention "bien"), allowing entry into the rankings for financial support by the French Government (Allocation de Recherche du MENRT). I further hold a PhD in Molecular and Cellular Pharmacology, ULP, Strasbourg, FRANCE, earned in 1994;

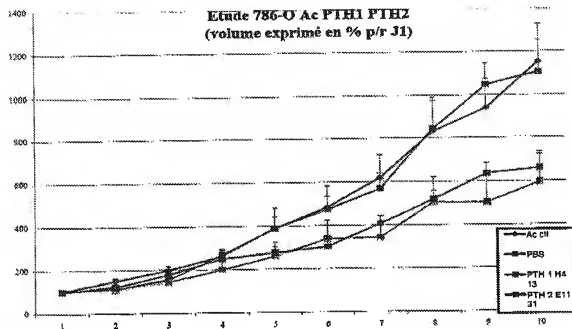
MASSFELDER et al.
10/520,085
Rule 132 DECLARATION

obtained with the highest possible distinction (mention "très honorable avec félicitations"), sparingly attributed by the ULP of Strasbourg. I earned a University Diploma: "Scientific manager in animal experimentation", in 2001, ULP (mention "assez bien").

4. I believe the above-identified application demonstrates, such as in Figures 4, 6 and 8, that an anti-PTHrP antibody directed against the intermediate or C-terminal regions of PTHrP, in particular anti-PTHrP (34-53) antibody and anti-PTHrP (107-139) antibody, decrease the proliferation of tumor cells.

5. To further demonstrate this effect I have produced, or had produced under my direction and control, two further distinct monoclonal anti-PTHrP (34-53) antibodies, called PTH1 H413 and PTH2 E1131 which demonstrated the important in vivo anti-tumoral effect of these antibodies. The following figure is a graph showing the percentage of tumoral volume in comparison with the volume of day 1 (day 1 is 100%) for two controls (PBS or non relevant antibody (AC cti) of the same antibody isotype than PTH1 and 2 and directed against Stra8, a protein expressed in male gonades and testis) (two upper curves) and the two anti-PTHrP (34-53) antibodies (two lower curves) administrated at 40 µg/mice each day. Mice weight is from 30-34 g. The tumoral volume has been measured every 3 and 4 days. Accordingly, measures 1 and 2 correspond to the Tuesday and the Friday of the first week, respectively and measures 3 and 4 correspond to the Tuesday and the Friday of the second week, and so on. Accordingly, the experiment lasts about 5 weeks. Antitumoral activity of these two antibodies has also been demonstrated in vitro on human renal cancer cells 786-0 and Caki-1.

MASSFELDER et al.
10/520,085
Rule 132 DECLARATION



6. The following describes the Tumor Model used in the investigation reported above. Seven-week-old male Swiss nu/nu nude mice (Iffa-Credo, St. Germain sur l'Arbresle, France) were given s.c. injections of 10 millions 786-O cells into the skin of the back. Mice weight is from 30-34 g. Tumor size was measured using calipers. Two weeks after injection, mice bearing tumors were separated each in four groups (6-8 mice each). Mice bearing tumors were injected i.p. daily with 40 µg of one of the two distinct monoclonal anti-PTHrP(34-53) antibodies (called PTH1 H413 and PTH2 E1131), with PBS or with the non relevant antibody. Tumor size was measured using calipers every 3-4 days. $P < 0.05$ PTH1 an 2 vs. Ac Ctl and PBS.

MASSFELDER et al.

10/520,085

Rule 132 DECLARATION

7. I believe the above results further demonstrate the utility of administering an anti-PTHrP antibody directed against an intermediate region of PTHrP to treat kidney cancer.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this 15th day of February, 2008.

(Signature) _____


Thierry MASSFELDER

Court of Customs and Patent Appeals

In re SMITH

No. 8590 Decided May 18, 1972

PATENTS

1. Board of Appeals — Procedure and practice (§19.45)

Pleading and practice in Patent Office — Rejections (§54.7)

Board is empowered under Patent Office Rule 196(b) to make new rejections; while Board should have labelled its rejection on reconsideration a new one, applicant did not seek to avail himself of rights accorded under Rule nor does he indicate any desire to further argue the rejection before Patent Office or to introduce additional evidence; accordingly, rejection is treated as before court on appeal.

2. Specification — Sufficiency of disclosure (§62.7)

Rule that disclosure of a genus and a species of a subgenus is a sufficient description of the subgenus is not consonant with description requirement of 35 U.S.C. 112; precisely how close the description must come to comply with section 112 must be left to case-by-case development; it cannot be said that subgenus is necessarily always implicitly described by a genus encompassing it and a species upon which it reads; to the extent that In re Risse, 154 USPQ 1, provides aforementioned rule for satisfaction of description requirement of first paragraph of section 112, it is overruled.

3. Claims — Broad or narrow — In general (§20.21)

There is nothing inherently wrong with a particular principle of patentability which under certain circumstances operates to defeat patentability of a narrow, but not a broader, claim, and, ordinarily, fact that under such a principle a broader claim would pass muster is no basis for adjusting principle to render narrower claim patentable.

Particular patents—Paint

Smith, Glossy Emulsion Coating Compositions Containing Surface Treated Pigments of Oleophilic Nature and Method, claims 1, 2, 12 to 18, 20, and 21 of application refused.

Appeal from Board of Appeals of the Patent Office

Application for patent of Richard G. Smith, Serial No. 430,468, filed Feb. 4, 1965; Patent Office Group 140. From decision rejecting claims 1, 2, 12 to 18, 20, and 21, applicant appeals. Affirmed.

HERBERT B. KEIL, RICHARD L. JOHNSTON, and JOHNSTON, ROOT, O'KEEFE, KEIL, THOMPSON & SHURTLEFF, all of Chicago, Ill., for appellant.

S. WM. COCHRAN (FRED E. MCKELVEY of counsel) for Commissioner of Patents.

Before RICH, ALMOND, BALDWIN, and LANE, Associate Judges, and RAO, Judge, United States Customs Court, sitting by designation.

LANE, Judge.

This appeal is from the decision of the Board of Appeals sustaining the examiner's rejection of claims 1, 2, 12-18, 20 and 21 of appellant's application, Serial No. 430,468, filed February 4, 1965, for "Glossy Emulsion Coating Compositions Containing Surface Treated Pigments of Oleophilic Nature and Method." This application is asserted to be a continuation of an application filed in 1956,¹ which was a continuation-in-part of a 1951 application,² which in turn was a continuation-in-part of Serial No. 774,897, filed September 18, 1947. We affirm the board's decision.

The Invention

The invention is directed to the compounding of a glossy water base emulsion paint. In his brief before this court, appellant distinguishes between single phase, oil base paints and dual phase, water base emulsion paints wherein there is a continuous phase of water in which globules of oil are suspended. By "oil" is meant "those natural and synthetic fluid organic water insoluble compounds commonly used as a whole or part of the vehicle or binder in coating compositions." Whereas oil base paints are said to be naturally glossy, water base emulsion paints tend to be "flat." Various advantages are claimed for water base paints, and the object of this invention was to produce a glossy water base emulsion paint.

Appellant postulates that the reason for flatness in a water base paint is "the fact that it contains two phases, the water phase preferentially wetting some of the pigment so that some pigment is contained in the volatile water phase." The concept underlying the present invention was the appreciation that "if the

¹ Serial No. 574,988 filed March 30, 1956.

² Serial No. 230,841 filed June 9, 1951.

in view of Martin² or o³ considered with Weller⁴ in discloses a metalizing paint using micro-circuits which contain metals, i.e. silver, gold, the metals and mixtures thereof."

Rao discloses an enamel composition containing (1) silver, in the ionic state, (2) finely divided platinum, palladium or mixtures thereof and (3) finely s of other noble metals, such as Weller relates to a method for inial leads in composite electrical on the ceramic body may y alloying, coating, or plating the lead with a material which migration." Finally, Short d isy problem of silver migration conductors or electrodes printed elements which is of concern is that problem "by interposing : between silver conductors of ity a barrier composed of a pium the group consisting of gold, cadmium."

t the claims were unpatentable

rt states that a surface coating of ill inhibit migration of silver in a moist atmosphere. Weller ver migration can be avoided by ating or plating the surface of with a material which inhibits ion, i.e., the inhibitor may be a ating or it may be distributed the body of the silver. In our is then obvious to workers of or l in this art to alloy or mix Pt with a silver conductor, for the inhibiting migration of silver. tion of optimum proportions of metals is a routine matter, based of the metals and degree of desired.

iew, the nuances between the ret matter and the prior art are ppellant's compositions would een obvious to the person of or in the art. The decision is there-

ent No. 3,293,501, issued December

ent No. 3,154,503, issued October 27,

ent No. 3,021,589, issued February

ent No. 2,758,267, issued August 7,

pigment used in these paints could be surface coated in such a way that it would be wholly wetted by the oil phase and not be permitted to migrate into the water phase," the problem would be solved.

The claims reflect this approach. Claims 1 and 2 are drawn to an emulsion coating composition having a continuous water phase and a discontinuous oil phase dispersed therein. The pigment is dispersed in the discontinuous oil phase and is maintained in that phase as a result of a surface coating of an organic compound which renders the pigment "oilophilic," i.e., having an affinity for oil, and particularly, having a preferential affinity for oil as compared to water. The organic compounds with which the pigment may be coated are described in the specification as follows:

[They] are monomeric organic compounds characterized by at least one non-polar organic hydrophobic group containing at least eight carbon atoms in a hydrocarbon structure which in the form of its monocarboxylic acid is soluble in oleoresinous varnishes and insoluble in water, and at least one polar group preferably selected from the class consisting of carboxylic acids, salts of said carboxylic acids, esters of said carboxylic acids and cationic ammonium and amine surface active groups containing an ionizable negative radical of a water soluble acid. Such polar groups are effective in causing said organic compounds to adhere to the pigment surface, especially where the latter are hydrophilic.

Claim 1, subdivided for convenience, reads as follows:

1. An emulsion coating composition comprising essentially
a continuous aqueous phase and
a discontinuous water insoluble oil phase containing dispersed in said discontinuous phase a pigment which is surface coated with an organic compound effective to render said pigment oilophilic,

said organic compound being a monomeric organic compound characterized by
at least one non-polar organic hydrophobic group containing at least 8 carbon atoms in a hydrocarbon structure, which group in the form of its monocarboxylic acid is soluble in oleoresinous varnishes and insoluble in water,

and at least one polar group,
said organic compound adhering to said pigment surface when said coated pigment is emulsified,

said coating having been applied to said pigment prior to emulsification thereof and

prior to dispersion of said pigment in said oil phase.

and said discontinuous pigmented phase being capable of forming a continuous solid glossy film when dried.

Claim 2 further defines the organic compound by the general formula:



where R is an organic radical containing 8 to 36 carbon atoms in a hydrocarbon structure, X is an inorganic cation, and n is a whole number from 1 to 2 * * *

A series of composition claims more limited with respect to the pigment and the organic compound with which it is coated were allowed by the examiner.

The process of making the composition is also claimed. Claim 12, which is representative of the process claims on appeal, reads in pertinent part as follows:

12. A process of preparing emulsion coating compositions comprising essentially a continuous aqueous phase and a discontinuous water insoluble oil phase containing a pigment dispersed in said discontinuous phase which comprises surface coating a pigment with an organic compound effective to render said pigment oilophilic and adding water to said pigment, thereafter adding an oil phase and emulsifying said surface coated pigment to form a water-in-oil, pigment-in-oil emulsion and converting said water-in-oil, pigment-in-oil emulsion to a pigment-in-oil, oil-in-water emulsion * * *.

The remaining process claims, 13-18, 20 and 21, define the method in varying degrees of specificity. Several process claims limited to a specific pigment and organic compound were allowed.

The References

Baldwin³ discloses modifying the surface energy characteristics of a pigment by "causing precipitated suspensions of water repellant metallic organic compounds to become adherent upon the surface of the pigment in the form of a thin film covering each pigment particle." Among the coating compounds disclosed are those used by appellant. The patentee was primarily concerned with improving pigments for use in a variety of systems, but specific advantages of pigments made by the disclosed method are stated to be "smoother and glossier paints * * *."

Iliff et al. (Iliff),⁴ a patent issued on May 4,

³ Patent No. 1,946,054 issued February 6, 1934.

⁴ Patent No. 2,440,953.

173 USPQ

173 USPQ

In re Smith

681

dispersion of said pigment in said
 uid discontinuous pigmented phase
 pable of forming a continuous solid
 m when dried.
 rther defines the organic compound
 ral formula:



R is an organic radical containing
 carbon atoms in a hydrocarbon
 ; X is an inorganic cation, and n is
 number from 1 to 2 ***

f composition claims more limited
 et to the pigment and the organic
 with which it is coated were al-
 re examiner.

cess of making the composition is
 d. Claim 12, which is representa-
 process claims on appeal, reads in
 art as follows:

a process of preparing emulsion
 compositions comprising essentially
 uous aqueous phase and a discon-
 water insoluble oil phase contain-
 nt dispersed in said discontinuous
 hich comprises surface coating a
 with an organic compound effective
 said pigment oilophilic and adding
 said pigment, thereafter adding an
 se and emulsifying said surface
 pigment to form a water-in-oil,
 in-oil emulsion and converting said
 -oil, pigment-in-oil emulsion to a
 in-oil, oil-in-water emulsion ***
 ning process claims, 13-18, 20 and
 the method in varying degrees of
 Several process claims limited to a
 gment and organic compound were

The References

discloses modifying the surface
 racteristics of a pigment by "caus-
 tated suspensions of water repellent
 rganic compounds to become ad-
 n the surface of the pigment in the
 thin film covering each pigment
 Among the coating compounds dis-
 those used by appellant. The pat-
 ricularly concerned with improving
 or use in a variety of systems, but
 vantages of pigments made by the
 method are stated to be "smoother
 r paints ***"

L. (Lliff),⁴ a patent issued on May 4,

1948, on an application filed April 11, 1944,
 involves aqueous emulsion coating composi-
 tions. Patentees state:

[D]ifficulties are encountered in produc-
 ing materials which produce glossy films or
 coatings and which possess satisfactory
 stability in this respect. The difficulty ap-
 pears to be due to a tendency for the white
 pigment (titanium dioxide) to migrate from
 the oil phase into the external aqueous
 phase of the emulsion resulting in instabili-
 ty with respect to producing glossy films or
 coatings.

This invention therefore presents as the
 principal object means for producing stable
 white or tinted emulsion coating composi-
 tions which will produce glossy films.

These objects are accomplished in the
 present invention by incorporating, as the
 principal pigment in the composition, tita-
 nium dioxide which has been treated (as
 hereinafter described) to impart to it or-
 ganophilic properties with respect to its
 action in the aqueous resin emulsion ve-
 hicle.

Lliff et al. refer to various methods of treating
 the pigments already in the prior art as of the
 time their application was filed which essen-
 tially involve coating the pigments with inor-
 ganic compounds, but also disclose that "other
 treatments which may impart organophilic
 properties to titanium pigments are not to be
 precluded from the present invention."

Cassel³ relates to improvements in the
 dyeing of textile fabrics with pigments where-
 in the problem of pigment adherence to such
 fabrics is solved by the addition of controlled
 quantities of a "pigmented lacquer-in-water
 emulsion" to the fabrics. In example 3 of the
 Cassel patent the emulsion is prepared by
 forming an aqueous "pulp" of blue pigment,
 adding it to an alkyd resin-in-oil solution to
 give a water-in-lacquer emulsion, and invert-
 ing the emulsion by pouring it into an aqueous
 solution of sodium lauryl sulfate to give a
 lacquer-in-water emulsion. The solicitor
 summarizes this process as comprising the
 sequence of mixing an untreated pigment with
 water, adding oil to give a water-in-oil emul-
 sion, and adding water and emulsifier to con-
 vert the water-in-oil emulsion to an oil-in-
 water emulsion.

The examiner additionally relied upon
 patents to Berry⁶ and Machlin,⁷ but the

board dismissed these as cumulative. The
 solicitor agrees that they are not before us,
 and we do not consider their teachings.

The Grounds of Rejection

In its original decision, the board treated
 the method claims as standing or falling with
 the composition claims and held all of the
 claims on appeal to be unpatentable under 35
 U.S.C. 103 over Lliff in view of Baldwin.
 Appellant requested reconsideration urging
 that the claimed method was patentably dis-
 tinct from the composition and should be
 treated separately. The board did reconsider
 its position and concluded that "except for the
 specific pigment-coating step," Cassel, a refer-
 ence previously applied by the examiner but
 dropped in the examiner's answer, "shows the
 manipulative steps of claim 12." A fair read-
 ing of the board's decision on reconsideration
 indicates that it was of the view that the sub-
 stitution of the claimed coated pigment, be-
 lieved to have been suggested by Lliff and
 Baldwin, for the untreated pigment used in the
 Cassel process would have been obvious to one
 of ordinary skill in the art.

[1] Appellant asserts that inasmuch as the
 examiner dropped Cassel, it is not before us.
 The difficulty with appellant's position is that
 the board is empowered under Patent Office
 Rule 196(b) to make new rejections, and we
 think it clearly did make a new rejection of the
 process claims as unpatentable under 35
 U.S.C. 103 over Cassel in view of Lliff and
 Baldwin. While the board should have la-
 belled its rejection on reconsideration a new
 one, appellant did not seek to avail himself of
 the rights accorded under Rule 196(b), nor
 does he now indicate any desire to further ar-
 gue the rejection before the Patent Office or to
 introduce additional evidence. We accordingly
 treat Cassel, and the rejection in which it is
 relied upon, as before us. See *In re Hyson*, 59
 CCPA ____, 453 F.2d 764, 766, 172 USPQ
 399, 401 (1972); *In re Cavrich*, 59 CCPA
 ____, 451 F.2d 1091, 1093, 172 USPQ 121,
 122 (1971). Giving due consideration to the
 time and effort which has already been ex-
 pended by the parties and this court to this
 point in the appeal, we do not feel that justice
 would be served by a remand which would in
 all probability fail to significantly aid anyone.

Opinion

To reiterate, we have two rejections before
 us in this appeal. The first is the rejection of
 composition claims 1 and 2 under 35 U.S.C.
 103 as obvious from Lliff in view of Baldwin.
 The other is the rejection of method claims 12-
 18, 20 and 21 under 35 U.S.C. 103 as obvious

³ Patent No. 2,343,642 issued February 29, 1944.
⁴ U.S. Pat. 2,394,521 issued February 26, 1946.

I. Availability of *Iliff* as a Reference

A threshold issue common to both rejections is the availability of *Iliff* as a reference. The *Iliff* patent was issued on May 4, 1948, on an application filed April 11, 1944. As noted above, appellant alleges that the application on appeal is in a chain of continuing applications, the earliest of which was filed on September 18, 1947, followed by one filed on June 9, 1951. During the course of prosecution of his prior applications, appellant submitted affidavits under Rule 131 purporting to prove conception and reduction to practice of the claimed invention prior to the effective date of *Iliff*.

The examiner attacked the sufficiency of the affidavits and also held that the 1947 application failed to support the presently claimed invention thereby depriving appellant of the right to rely upon its filing date. Since *Iliff* issued more than one year prior to the filing date of the 1951 application, the examiner concluded that *Iliff* was statutory bar and could not be overcome by a Rule 131 showing.⁸ The board agreed that *Iliff* is a statutory bar and did not, therefore, give much consideration to the substance of the affidavit evidence. Concerning the 1947 application, the board stated:

We find no mention in that application of the requirement that the coating compound must be a monomer having at least 8 carbon atoms in its hydrophobic moiety, and that more than one polar group was contemplated, all of which is recited in claim 1. With respect to claim 2, we find no disclosure in the above parent [1947] application of the 8 to 36 atom limitation, or the specific presence of 2 carboxylate groups in the coating compound. While the questioned limitations are consistent with the broader aspects of the parent disclosure, they could not be specifically claimed in the parent application. [Emphasis in original.]

Appellant asserts that the 1947 application contains a disclosure of organic coating compounds which is generic to the compounds which appear in the present claims as well as a disclosure of species upon which the subgenus of organic compounds now claimed reads. This, he argues, is sufficient for a supporting parent disclosure relying upon *In re Kisse*,

⁸ Rule 131 provides that a patent will not bar the grant of a patent to an applicant when it is proved that the invention sought to be patented was completed prior to the reference patent filing date "unless the date of such patent . . . be more than one year prior to the date on which the application was filed in this country." See 35 U.S.C. 102 (b).

54 CCPA 1495, 378 F.2d 948, 154 USPQ 1 (1967). He points out the following portion of the 1947 specification:

The treatment of pigments with polar agents is not new per se and can be accomplished by several methods employing a variety of effective compounds. In general these methods involve surface coating the pigment with an oil soluble polar organic compound. Among the polar organic compounds are acidic resins, water soluble resins, long chain fatty acids, their salts and soaps, benzene carboxylic acid and its salts, naphthene acids and their soaps and salts, cationic active agents, e.g., alkyl amine salts and quaternary ammonium compounds containing at least 12 carbon atoms in an alkyl group or groups, e.g., lauryl pyridinium bromide, and long chain (at least 12 carbon atoms) fatty acid-containing organic Werner complexes.

Comparing this disclosure to the compounds recited in the claims on appeal, appellant contends:

It is obvious that the surface coating organic compounds recited in the foregoing paragraph are monomeric, have a hydrocarbon structure of at least 8 carbon atoms, except for benzene carboxylic acid which contains six carbon atoms in a hydrocarbon group, and contain at least one carboxy or carboxylate group. If appellant's claims had been drawn more broadly, they would be supported by the parent application. They can be described as subgeneric claims because they delineate the invention more specifically by reciting that the organic material used to coat the pigment is monomeric, contains at least 8 carbon atoms and at least one carboxy or carboxylate group.

The principal question involved is whether or not appellant is entitled under 35 U.S.C. 120 to the benefit of the filing date of his 1947 application for the subject matter presently claimed.⁹ To comply with § 120, the prior application must satisfy "the disclosure requirements of the first paragraph of § 112 . . . with respect to the subject matter now

⁹ We note that the 1947 application was abandoned in 1951, prior to the January 1, 1953, effective date of title 35, United States Code. See Act of July 19, 1952, ch. 950, § 4, 66 Stat. 815, reproduced at 35 U.S.C. at 71. The solicitor nevertheless asserts that 35 U.S.C. 120 governs, and we agree. The 1951 application was pending in 1953, and according to the Act of July 1952, § 4, title 35 applied to pending applications. Thus, appellant can rely on 35 U.S.C. 120 for the effective date of his next preceding 1947 application if the requirements of § 120 are otherwise satisfied.

1495, 378 F.2d 948, 154 USPQ 1
e points out the following portion of
pecification:

treatment of pigments with polar
s not new per se and can be accom-
by several methods employing a
of effective compounds. In general
methods involve surface coating the
: with an oil soluble polar organic
nd. Among the polar organic com-
are acidic resins, water soluble
s, water insoluble metallic resin-
g chain fatty acids, their salts
ps, benzene carboxylic acid and its
phenic acids and their soaps and
ationic active agents, e.g., alkyl
salts and quaternary ammonium
nds containing at least 12 carbon
n an alkyl group or groups, e.g.,
yridinium bromide, and long chain
12 carbon atoms) fatty acid-con-
organic Werner complexes.

t this disclosure to the compounds
he claims on appeal, appellant con-

bviously that the surface coating or-
ncompounds recited in the foregoing
h are monomeric, have a hydro-
: structure of at least 8 carbon atoms,
r benzene carboxylic acid which
six carbon atoms in a hydrocarbon
nd contain at least one carboxy or
ate group. If appellant's claims had
wn more broadly, they would be
f by the parent application. They
scribed as subgeneric claims be-
y delineate the invention more
ly by reciting that the organic ma-
d to coat the pigment is monomer-
s at least 8 carbon atoms and at
carboxy or carboxylate group.

tional question involved is whether
llant is entitled under 35 U.S.C.
enefit of the filing date of his 1947
for the subject matter presently
o comply with § 120, the prior
must satisfy "the disclosure re-
of the first paragraph of § 112
respect to the subject matter now
that the 1947 application was aban-
prior to the January 1, 1953, effec-
35, United States Code. See Act of
ch, 950, § 4, 66 Stat. 815, reproduced
71. The solicitor nevertheless asserts
120 governs, and we agree. The 1951
is pending in 1953, and according to
1952, § 4, title 35 applied to pending
hus, appellant can rely on 35 U.S.C.
ective date of his next preceding 1947
the requirements of § 120 are other-

173 USPQ

claimed." Martin v. Johnson, 59 CCPA
454 F.2d 746, 172 USPQ 391 (1972); In
Brower, 58 CCPA 724, 433 F.2d 813, 167
USPQ 684 (1970). The examiner's and
board's refusal to accord appellant the benefit
of the filing date of his 1947 application was
premised on their finding that the invention
now claimed was not "disclosed" in, or
"supported" by, the 1947 disclosure. Trans-
lated in terms of a first paragraph, § 112,
requirement, it is evident that the Patent Of-
fice holding was that there was no description
of the invention now claimed in the earlier
specification. See Martin v. Johnson, supra;
In re Lukach, 58 CCPA 1233, 442 F.2d 967,
169 USPQ 795 (1971).

In re Risse, supra, relied upon by appellant,
involved, inter alia, the question of "support"
for a claimed subgenus in the disclosure of a
parent application. The court did not speak in
terms of the description requirement of § 112,
and it is since Risse that this court has focused
on the express language of the statute. The
recent cases suggest a more stringent re-
quirement for a description of the claimed in-
vention than may have been previously ap-
plied in cases wherein the issue was framed in
terms of "support" for claimed subject matter.
Compare Martin v. Johnson, supra; Fields v.
Conover, 58 CCPA 1366, 443 F.2d 1386, 170
USPQ 276 (1971); In re Lukach, supra; In re
DiLeone, 58 CCPA 934, 436 F.2d 1033, 168
USPQ 598 (1971); In re DiLeone and Lucas,
58 CCPA 925, 436 F.2d 1404, 168 USPQ 592
(1971); In re Ahlbrecht, 58 CCPA 848, 435
F.2d 908, 168 USPQ 293 (1971); with Risse,
supra; In re Grimme, 47 CCPA 785, 274 F.2d
949, 124 USPQ 499 (1960). Doubt as to the
continuing vitality of Risse in light of the sub-
sequent "description requirement" cases has
already been cast in Lukach and Fields v.
Conover.

In distinguishing In re Fried, 50 CCPA
954, 312 F.2d 930, 136 USPQ 429 (1963),
and Watson v. Bersworth, 251 F.2d 898, 116
USPQ 79 (D.C. Cir. 1958), this court in Risse
said:

The critical distinction is that in the
Fried and Watson v. Bersworth cases, each
of the applicants was attempting to claim a
subgenus not specifically disclosed as such
in the parent case, which contained only
generic disclosure but no description of a
single species within the scope of the later
claimed subgenus. It is difficult to arrive at
such a subgenus by a purely deductive ap-
proach, selecting appropriate variables from
the generic disclosure. On the other hand,
one may more easily reach such a subgenus
by proceeding toward it from two opposite

directions, i.e., by an inductive approach
from a specifically disclosed species within
the subgenus, as well as the deductive ap-
proach from the generic disclosure. The
latter situation is represented by the facts of
this case as well as Grimme. In both cases
the subgeneric claims of the continuation-
in-part applications (1) are completely
within the scope of the parent case generic
disclosure and (2) read on at least one spe-
cies disclosed in a working example of the
parent application.¹⁰

[2] From this passage emerges the rule re-
lied upon by appellant to the effect that the
disclosure of a genus and a species of a
subgenus is a sufficient description of the
subgenus. We do not now feel that such a rule
is consonant with either the letter or spirit of
the description requirement of § 112.

Precisely how close the description must
come to comply with § 112 must be left to
case-by-case development. However, the rule
extracted from Risse is much too broad.
Whatever may be the viability of an inductive-
deductive approach to arriving at a claimed
subgenus, it cannot be said that such a
subgenus is necessarily always implicitly de-
scribed by a genus encompassing it and a spe-
cies upon which it reads. Unfortunately, Risse
has been interpreted as so saying, and to put
the proposition to rest, we overrule Risse to
the extent that it provides the aforementioned
"rule" for the satisfaction of the description
requirement of the first paragraph of § 112.

[3] Appellant apparently perceives an
anomaly resulting from the failure to follow
Risse when he contends that had he simply
drawn his claims more broadly, he would have
satisfied the requirements of § 112. However,
even assuming that broader claims would have
descriptive support, it does not follow that
such claims would be otherwise patentable.
Section 112 itself imposes several conditions
on the disclosure and claims, and the per-
tinance of the three prerequisites to patent-
ability of a given claimed invention—utility,
novelty and nonobviousness—may well de-
pend upon its breadth. We see nothing inher-
ently wrong with a particular principle of
patentability which under certain circum-
stances operates to defeat the patentability of a
narrow, but not a broader, claim, and, ordi-
narily, the mere fact that under such a prin-
ciple a broader claim would pass muster is not
a basis for adjusting the principle to render the
narrower claim patentable.¹¹ We will not at-

¹⁰ 54 CCPA at 1500, 378 F.2d at 952, 154 USPQ
at 5.

¹¹ But see In re Stryker, 58 CCPA 797, 435 F.2d
1340, 168 USPQ 772 (1971).

tempt to anticipate the patentability of claims different in scope from those on appeal as a step antecedent to deciding a particular issue as applied to the particular claims before us.

Without the Risse doctrine, appellant has no basis on which the disclosure in the 1947 application may be treated as a description of the subject matter now claimed. The situation resembles that in *Ahlbrecht* wherein the subgenus sought to be patented was actually an extension of the only subgenus specifically described in the prior application. In *Ahlbrecht*, we held that the parent disclosure of fluorinated esters having "m" number of CH₂ groups, wherein "m" was disclosed as an integer from 3 to 12, was not a legally sufficient description of subsequently claimed esters wherein "m" was an integer from 2 to 12. Hence, even accepting the thrust of appellant's contention to the effect that the subgenus of pigment coating compounds now claimed encompasses compounds that were disclosed in the 1947 application, we nevertheless find ourselves in agreement with the board and the solicitor that the claimed subgenus of coating compounds with at least 8 carbon atoms was not adequately described in the earlier application which disclosed compounds with at least 12 carbons.

Since the 1947 application does not describe the claimed subject matter in the contemplation of § 112, first paragraph, appellant is not entitled to the § 120 benefit of the filing date of that earlier application. Accordingly, *Iliff* stands as a § 102 (b) statutory bar available for consideration along with other prior art in the determination of obviousness under 35 U.S.C. 103, and appellant may not, by Rule 131 affidavit, overcome it.

II. Obviousness of the Composition

The board held that it would have been obvious to substitute Baldwin's coated pigment for the coated pigment disclosed by *Iliff*. Appellant asserts that such a substitution would not have been obvious since Baldwin is concerned with oil base paints whereas *Iliff* involves water base paints. The essence of the argument would appear to be that the two environments are so dissimilar that one of ordinary skill in the art would not extract from Baldwin a teaching applicable to water emulsion paints.

The solicitor stresses that *Iliff* recognized the problem of low gloss value in emulsion paints, its source in the tendency of pigments to migrate from the oil to the water phase, and the conceptual solution of coating the pigment to impart nonaqueous properties. The solicitor

praises applying an inorganic coating, and concludes that there is ample suggestion to employ the Baldwin coating which is taught to yield glossier paints.

We agree with the conclusion of the board and the solicitor's reasoning. In *Iliff*, we find a recognition of the problem faced by appellant and its general solution. *Iliff* appreciated that treatments other than coating with an inorganic material would be suitable. Baldwin discloses such an alternative treatment, and we believe that given the direction provided in *Iliff*, one of ordinary skill in the art would be led to employ Baldwin's coatings in lieu of *Iliff*'s. As taught by both *Iliff* and Baldwin, the use of such coated pigments would yield glossier paints, the result contemplated by appellant.

We affirm the rejection of composition claims 1 and 2 under 35 U.S.C. 103.

III. Obviousness of the Method

The board regarded the sole difference between Cassel and the presently claimed process to be the pigment-coating step. Having concluded that coating a pigment with the organic compound claimed would have been obvious from *Iliff* and Baldwin, the board considered the claimed method obvious. Appellant argued before the board that the affidavit of Willis demonstrated that the results of the claimed process are superior to those achieved by Cassel. Specifically, affiant compared the gloss of a coating of an emulsion prepared by the method described in example 3 of the Cassel patent to that of a coating of an emulsion prepared by the claimed process and found the former to be much poorer. The board did not regard the Willis affidavit to be persuasive of nonobviousness saying:

Appellant refers to the Willis affidavit filed October 23, 1967, in which Example 3 of Cassel * * * was duplicated and allegedly did not produce appellant's result of a glossy coat. However, it appears from the affidavit that the Cassel method did produce a water-continuous emulsion by inventing an oil-continuous emulsion prepared by flushing an aqueous pigment dispersion into an oil vehicle; i.e., except for the specific pigment-coating step, Cassel shows the manipulative steps of claim 12.

Before this court, appellant notes that his specification states that "[i]nitially to disperse a pigment in water that one desires to be dispersed in oil is a novel concept which would not be done deliberately to bring a pigment into an oil phase," and urges that Cassel fails to suggest this discovery. The solicitor

173 USPO

inorganic coating, and is a simple suggestion to coating which is taught to

the conclusion of the board of appeal. In *Illiff*, we find a problem faced by appellant *Illiff* appreciated that an coating with an inorganic coating would be suitable. Baldwin's inventive treatment, and we direct the board in *Illiff* in the art would be led to coatings in lieu of *Illiff*'s, *illiff* and Baldwin, the use of which would yield glossier results than the glossier contemplated by appellant. *Illiff* 35 U.S.C. 103.

Conclusion of the Method

led the sole difference between the present claimed process-coating step. Having a pigment with the oil-armed would have been and Baldwin, the board method obvious. Appeal the board that the affidavit shows that the results of the process are superior to those of the prior art. Specifically, affiant compares a coating of an emulsion with the method described in example 1 to that of a coating of an oil-armed pigment by the claimed process and the results are much poorer. The board in the *Willis* affidavit to be obvious saying:

As to the *Willis* affidavit 1967, in which Example 3 is duplicated and allegedly appellant's result of a glossier, it appears from the affidavit method did produce a glossier emulsion by inverting an emulsion prepared by flush-pigment dispersion into an accept for the specific pigment. Cassel shows the manipulation 12.

Appellant notes that his art "initially to disperse that one desires to be dissolved concept which would be to bring a pigment and urges that Cassel fails to show. The solution comes

173 USPO

coated pigment such as Baldwin's would use the Cassel method expecting that because of its increased affinity for oil and decreased affinity for water, the coated pigment would ultimately end up in the oil phase.

We think the Patent Office has made out a strong prima facie case of obviousness of the claimed method. It appears that in terms of a sequence of manipulative steps, Cassel discloses the method herein claimed. Although Cassel utilizes an untreated pigment, we agree that the substitution of the coated pigment taught by *Illiff* and Baldwin therefore would be prima facie obvious.

The *Willis* affidavit does not provide a persuasive rebuttal of the prima facie case. While appellant interprets the affidavit showing as proof that Cassel's method fails to yield a glossy emulsion, the comparison would appear to turn on the difference in the pigments employed. It is expected from the disclosures in *Illiff* and Baldwin that a coated pigment will produce a glossy emulsion whereas an untreated pigment will not. In short, the proven distinction between the Cassel disclosure and the claimed method is only the expected one, and as such, it is not persuasive of nonobviousness. We accordingly affirm the rejection of claims 12 through 18, 20 and 21.

The decision of the board sustaining the rejection of all the claims here on appeal is affirmed.

Court of Customs and Patent Appeals

In re BROWN AND SAFFER

No. 8621

Decided May 18, 1972

PATENTS

1. Court of Customs and Patent Appeals — Briefs (\$28.05)

Court does not consider specific references cited in solicitor's brief since these references were not relied on in rejection at bar.

2. Claims — Article defined by process of manufacture (§20.15)

Patentability — Subject matter for patent monopoly — Process, product and apparatus (§51.613)

In order to be patentable, product must be novel, useful and unobvious; this is true

In re Brown and Saffer

685

this latter type of claim, called a product-by-process claim, does not inherently conflict with second paragraph of 35 U.S.C. 112; that method of claiming is acceptable so long as claims particularly point out and distinctly claim product or genus of products for which protection is sought and satisfy other requirements of statute; however, lack of physical description in product-by-process claim makes determination of patentability more difficult, since it is patentability of product claimed and not of recited process steps which must be established; therefore, when prior art discloses a product which reasonably appears to be identical with or only slightly different than product claimed in product-by-process claim, a rejection based on sections 102 or 103 is fair; Patent Office is not equipped to manufacture products by myriad of processes put before it and then obtain prior art products and make physical comparisons therewith.

Particular patents—Catalyst

Brown and Saffer, Ethylene Oxidation Catalyst, claims 7 to 14 of application allowed; claims 15 to 19 refused.

Appeal from Board of Appeals of the Patent Office.

Application for patent of David Brown and Alfred Saffer, Serial No. 612,731, filed Jan. 30, 1967; Patent Office Group 113. From decision rejecting claims 7 to 19, applicants appeal. Affirmed as to claims 15 to 19; reversed as to claims 7 to 14.

WILLIAM C. LONG and DAVID DICK, both of New York, N. Y., for appellants.
S. WM. COCHRAN (FRED W. SHERLING for counsel) for Commissioner of Patents.

Before RICH, ALMOND, BALDWIN, and LANE, Associate Judges, and RAO, Judge, United States Customs Court, sitting by designation.

BALDWIN, Judge.

This appeal is from the decision of the Patent Office Board of Appeals affirming the examiner's rejection of claims 7-19 in appellant's application.¹ No claims have been allowed.

The Invention

The invention relates to catalysts for vapor phase partial oxidation of ethylene to ethylene oxide. Claims 7 and 15, the only independent claims in the case, read as follows:

168 USPQ

168 USPQ

In re Ahlbrecht

293

I, ALMOND, BALDWIN, and Associate Judges, and NEWMAN, United States Customs Court, signation.

udge.

It is from the decision of the Board of Appeals affirming the rejection of claims 4-8 and 12-15, 35 U.S.C. 103 as obvious in view of art.

tion relates to a three-component, perhaps best described as hot dog coated with a batter stuffing comprises such as, sauerkraut, peanut butter, or semi-solid food material, the specification, a layer of hamburger or sausage meat, completely surrounds the manner to contain and prevent portions of the inner stuffing. After the outer surface of the layer is covered with a batter, the product is deep fat fried, strait.

d product comprising:
ood component having a sub-cylindrical shape,

d food component consisting of comminuted meat surrounded by a layer of uniform thickness, and

usually uniform coating derived from and having a thickness of an magnitude of one eighth inch

product being substantially in the shape of a hot dog and having dimensions of magnitude no greater than the dimensions of a hot dog.

ices are:

1,492,603 May 6, 1924

1,591,945 July 6, 1926

1,706,491 Mar. 26, 1929

2,822,571 Feb. 11, 1958

in patent 127,632 April 30,

specification 786,217 Nov. 13,

closes a three-component food product for deep fat frying comprising a sausage entirely surrounded

outing, which in turn is coated by a layer of precooked potatoes, a hollow frankfurter with

in application serial No. 374,076, 1964 for "Food Product and Process"

an axial opening end-to-end into which various foods, such as cheese or relish, may be stuffed. The resultant product is placed in the conventional hot dog bun. Matson, Sawkins, Jenkins and Australian all disclose deep fat frying or baking batter-coated frankfurters or sausages.

The examiner rejected all claims under 35 U.S.C. 103 in view of the above references on several grounds. For one, he regarded appellant's three-component food product to distinguish from that of British principally in the particular order or arrangement of the product components, with the placement of one of the food components *inside*, rather than *outside*, the meat component to be no more than a matter of obvious choice. For another, he rejected the claims as unpatentable over Jenkins or Australian in view of Johnson or, alternatively, as unpatentable over Johnson in view of each of Matson, Sawkins, Jenkins or Australian. The crux of the examiner's position there seems to have been that it would be obvious to one of ordinary skill in the art to *completely* surround, even at the ends, the inner stuffing component of Johnson with the comminuted meat component, and subsequently coat the resulting product with a batter prior to deep fat frying, all as suggested by the remaining references.

The board, while preferring the rejection employing the Johnson, Jenkins and Australian references, agreed with the examiner's other stated grounds of rejection, and affirmed.

Appellant's principal argument here is that there is no factual basis to support an allegation that Johnson suggests *surrounding* a stuffing component with comminuted meat. It cannot be gainsaid, of course, that Johnson does not explicitly disclose *entirely* surrounding his stuffing component with comminuted meat, but contents himself with enclosing merely the sides of the cylindrical tube of stuffing, leaving the ends open. The examiner recognized as much, and turned to the Matson, Sawkins, Jenkins or Australian references for evidence that those of ordinary skill in the art would as a matter of course completely surround one food component with another as convenience, necessity, or choice in cooking or eating dictates.² We

²Such a matter of common knowledge is also well illustrated in the following recipe taken from Woman's Day Collector's Cook Book, Fawcett Publications, Inc., Greenwich, Connecticut, Copyright 1960, second printing June 1963, page 50:

Chicken Kiev

Thin chicken cutlets are wrapped around cubes of butter, then fried.

find no reversible error in that determination.

The view we take renders it unnecessary to consider the other grounds of rejection advanced below. The decision is *affirmed*.

Court of Customs and Patent Appeals

In re AHLBRECHT

No. 8417 Decided Jan. 7, 1971

PATENTS

1. Interference — Reduction to practice — Constructive reduction (\$41.755)

Specification — Sufficiency of disclosure (\$62.7)

35 U.S.C. 120 specifies only that previously filed application must disclose invention "in the manner provided by first paragraph of section 112"; there is no requirement under section 120 that invention claimed in subsequent application must correspond to what was regarded as the invention in earlier application.

2. Specification — Sufficiency of disclosure (\$62.7)

First paragraph of 35 U.S.C. 112 means that there must be both a written description in full, clear, concise, and exact terms, and that it enable any person skilled in the art to make and use the invention.

Particular patents—Esters

Ahlbrecht, Fluorinated Esters. claim 7 of application refused.

- 1 1/2 sticks butter
- 6 chicken breasts
- Fine dry bread crumbs
- 3 eggs
- 2 tablespoons cold water
- Vegetable shortening

Cut butter in half, lengthwise, then in twelve 2" pieces. Chill until very firm. Cut chicken breasts in half, remove bones. On wet board, pound chicken into thin cutlets. Put a piece of butter in center of each. Roll chicken around butter; fold securely so butter cannot escape during cooking. Secure with toothpicks. Roll in bread crumbs; dip in eggs beaten with cold water; roll again in crumbs. Fry in hot deep shortening (375° on a frying thermometer) 3 to 5 minutes. Drain, and put on cookie sheet in hot oven, 425° F., 5 minutes. Makes 6 servings.

Appeal from Board of Appeals of the Patent Office.

Application for patent of Arthur H. Ahlbrecht, Serial No. 520,087, filed Jan. 12, 1966; Patent Office Group 120. From decision rejecting claim 7, applicant appeals. Affirmed.

HAROLD J. KINNEY and CRUZAN ALEXANDER, both of St. Paul, Minn., and WALTER N. KIRN, Washington, D. C., for appellant.

S. WM. COCHRAN (FRED W. SHERLING of counsel) for Commissioner of Patents.

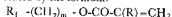
Before RICH, ALMOND, BALDWIN, and LANE, Associate Judges, and NEWMAN, Judge, United States Customs Court, sitting by designation.

ALMOND, Judge.

This is an appeal from the decision of the Patent Office Board of Appeals, adhered to on reconsideration, refusing to accord claim 7 in appellant's application¹ the benefit of an earlier filing date under 35 U.S.C. 120 and sustaining the rejection of that claim under 35 U.S.C. 102 as anticipated by the intervening patents to Langerak et al.² and Fasick et al.³ Claims 2-6 in the application stand allowed. We affirm.

The Invention

The esters of the claim in issue may be represented by the formula:



wherein R is a member of the group consisting of hydrogen and the methyl radical, R_f is a perfluoroalkyl radical having from 3-12 carbon atoms, and m is an integer from 2-12. Among several available methods, these acrylates may be prepared from ω -perfluoroalkyl-substituted alkanols of the formula,



in which R_f is the perfluoroalkyl group, by reacting the alkanol with acrylic or methacrylic acid of the formula,



in which R is a methyl radical in the case of

methacrylic acid or hydrogen in the case of acrylic acid.

Claim 7, the only one on appeal, broadly claims the esters produced by this process. It reads:

7. A perfluoroalkyl-alkyl acrylate or acrylic or methacrylate acid and an ω -perfluoroalkyl-substituted alkanol having not more than 12 methylene groups per molecule and in which the perfluoroalkyl radical has from 3 to 12 carbon atoms.

It is stated that the resulting unsaturated acrylates are useful as monomers to produce polymers by homopolymerization or by copolymerization with other vinyl monomers, and because of the perfluoroalkyl terminal group repeated along the polymer chain, when fibers of fabrics or textiles are coated with these polymers, oil and water resistance is imparted to the fibers. It is also stated that the polymers of the esters of this invention are useful in sheet form for such things as gaskets and cap liners, and show good resistance to oils.

Background

The examiner rejected claim 7 under 35 U.S.C. 102 as fully met by either Langerak et al. or Fasick et al., and appellant does not question the rejection of claim 7 over the references if the present application is not accorded the benefit of the filing date of application serial No. 677,229, filed August 9, 1957. Therefore, a detailed analysis of the references is not necessary.

The present application is derived from two chains of previously filed copending applications, both of which start with serial No. 677,229. Appellant contends that he is entitled to rely under 35 U.S.C. 120 on this parent application for an effective filing date of August 9, 1957, which date is sufficient to remove the references of record. The examiner took the position that serial No. 677,229 is inadequate to overcome the cited references because the invention described therein does not include esters having two methylene groups (i.e., where m in the formula is 2), but rather the description is confined to esters wherein m is 3-12.

[1] At this point, we note that a similar issue was recently presented in *In re Brower*, 167 USPO 684, Patent Appeal No. 8360, decided November 25, 1970. There we pointed out that 35 U.S.C. 120 specifies only that the previously filed application must disclose the invention "in the manner pro-

¹Serial No. 520,087 filed January 12, 1966, for "Fluorinated Esters."

²Patent No. 3,248,260 issued April 26, 1966, on an application filed August 22, 1961, which in turn was a continuation-in-part of an application filed May 2, 1960.

³Patent No. 3,282,905 issued November 1,

acid or hydrogen in the case of the only one on appeal, broadly esters produced by this process.

perfluoroalkyl-alkyl acrylate of α -methacrylate acid and an ω -alkyl-substituted alkanol having more than 12 methylene groups per and in which the perfluoroalkyl as from 3 to 12 carbon atoms.

ed that the resulting unsaturated useful as monomers to produce by homopolymerization or by co- with other vinyl monomers, e of the perfluoroalkyl terminal s along the polymer chain, s of fabrics or textiles are coated polymers, oil and water resistance to the fibers. It is also stated that s of the esters of this invention are eef form for such things as gaskets izers, and show good resistance to

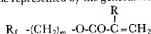
in the subsequent application must correspond to what was regarded as the invention in the earlier application. While this narrows the issue here, it is not determinative because the parties disagree as to whether the disclosure in the earlier application is sufficient under the first paragraph of 35 U.S.C. 112 to support the invention claimed in claim 7. In order to determine whether or not there is support in the parent application, the disclosure of the original specification must be considered.

The Parent Application

The tenor of the disclosure in the parent application, as it relates to the issue on appeal, is indicated by the following statement taken from the initial paragraphs of the specification:

This invention relates to fluorinated esters and more particularly to unsaturated aliphatic acid esters of ω -perfluoroalkyl-substituted alkanols.

The new polymerizable monomeric esters of unsaturated aliphatic acids included within the scope of the invention can be represented by the general formula:



wherein R is hydrogen or a methyl radical, R_1 is a perfluoroalkyl group having from 3 to 12 carbon atoms and m is an integer from 3 to 12. Also included within the scope of the invention are the polymers and copolymers of the above-described monomers. [Emphasis added.]

The remainder of the disclosure is similarly directed to "esters of terminally perfluorinated alcohols in which a chain or bridge containing at least three methylene groups is interposed between the perfluoroalkyl 'tail' and the hydroxyl group ***." [Emphasis added.]

Three methods are disclosed for preparing the perfluoroalkyl alkanol intermediate materials. Two of these methods cannot be used to prepare intermediate alkanols wherein m is 2, and thus the resulting esters cannot have two methylene groups. In regard to the third method, it is disclosed in the original specification that:

Other analogous addition reactions of perfluoroalkanesulfonyl halides to unsaturated esters and acids, as described in the copending application of G. V. D. Tiers, S.N. 532,743, filed September 6, 1955, followed by reductive dehydrohalogenation, also lead to the intermediate perfluoroalkyl alkanols of the present invention,

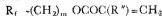
either by further saponification of the resulting esters or by reduction of the corresponding distally perfluoroalkylated aliphatic acids or esters thereof using, for example, lithium aluminum hydride.

The illustrations of this method in the parent disclosure show the production of alkanols wherein m is 4 and 10. However, as appellant points out in his brief, Tiers does disclose a starting material, vinyl acetate, which when reacted with a perfluoroalkanesulfonyl chloride and reductively dehydrohalogenated and saponified, results in the substituted alcohol $R_1-(CH_2)_mOH$, which can be used to produce esters wherein m is 2.

The Present Application

There is no dispute that the present specification supports the claim now on appeal as required by 35 U.S.C. 112. Appellant has altered the language of the specification to explicitly point out that esters having two methylenes are contemplated. For example, it is stated that:

*** The acrylate-type esters of the invention are prepared by esterifying the saturated alcohols with acrylic acid or methacrylic acid. These esters have the general formula:



wherein R_1 and m are as previously defined herein (i.e. R_1 is a perfluoroalkyl group having from 3 to 12 carbon atoms and m is 2-12, m being ordinarily 3-12) and R'' is hydrogen or methyl. [Emphasis added.]

While claim 7 is expressed in terms of an ester produced from "an ω -perfluoroalkyl-substituted alkanol having not more than 12 methylene groups per molecule," the examiner has agreed with appellant that the symbol omega implies that the alkanol has at least two methylene groups per molecule. Thus, the claim is directed to esters wherein m is 2-12, as disclosed in the present specification.

Opinion

The sole issue in the present appeal is whether the disclosure in the parent application complies with the first paragraph of 35 U.S.C. 112 for purposes of obtaining the benefit of the filing date of the parent application under 35 U.S.C. 120 so as to provide a basis for antedating the unquestionably anticipatory disclosures of Langerak et al. and Fasick et al. From our review of the complete disclosure of appellant's parent application, as outlined above, and a consideration of the cases cited and arguments advanced, we

Background

aminer rejected claim 7 under 35 as fully met by either Langerak sick et al., and appellant does not rejection of claim 7 over the re-he present application is not ac-benefit of the filing date of appli-it No. 677,229, filed August 9, efore, a detailed analysis of the not necessary.

ent application is derived from of previously filed copending apth of which start with serial No. appellant contends that he is en-y under 35 U.S.C. 120 on this eation for an effective filing date, 1957, which date is sufficient to references of record. The exam-e position that serial No. 677,229 e to overcome the cited references invention described therein does esters having two methylene where m in the formula is 2), e description is confined to esters 3-12.

is point, we note that a similar cently presented in In re Brower, 684, Patent Appeal No. 8360, vember 25, 1970. There we that 35 U.S.C. 120 specifies previously filed application must invention "in the manner pro-first paragraph of section 112," clear that there is no requirement n 120 that the invention claimed

agree with the Patent Office that the disclosure in the parent application is not sufficient to support claim 7.

[2] Paragraph 1 of § 112 provides that "The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same ***." This means that there must be both a written description in full, clear, concise, and exact terms, and that it enable any person skilled in the art to make and use the invention. While appellant's parent application may by reference to the copending application of Tiers contain enough to enable a person skilled in the art to make esters wherein m is 2, there is no explicit disclosure that m may be 2 or that certain starting materials from the Tiers disclosure may be used, which starting materials would ultimately lead to esters with two methylenes. That is, there is no description in full, clear, concise, and exact terms of esters wherein m is 2. The only esters described in such terms are those wherein m is 3-12.

Appellant relies heavily on the statements in the parent application that the invention relates particularly to esters of ω -perfluoroalkyl-substituted alkanols, and that the omega indicates that esters with two methylenes are included. We agree with the board that the reference to ω -perfluoroalkyl-substituted acrylate esters indicates only the specific field of art involved, and not that this is a class of esters all of which are disclosed. The omega merely indicates the position most removed from the functional group, and thus m may be anything over 2. However, appellant describes only the class of esters wherein m is 3-12. There is nothing in the original specification to indicate that any other esters (i.e., those where m is 2 or greater than 12) may be made by the methods disclosed or that they may be used as the disclosed esters. Therefore, it must be concluded that the only class of esters described in full, clear, concise, and exact terms is that wherein m is 3-12, and that the reference to ω -perfluoroalkyl-substituted acrylate esters is only a statement introducing the specific area of art involved.

This is unlike the situation in Brower, supra, where there was an explicit description of the subsequently claimed process, but there were statements to the effect that the part of the disclosure in question was not re-

garded as the invention, but rather here esters wherein m is 2 were never described in explicit terms at all.

Appellant's reliance on this court's decision in *In re Risse*, 54 CCPA 1495, 378 F.2d 948, 154 USPQ 1, 5 (1967), is misplaced. In *Risse*, where the parent supported its claims, a sufficient disclosure for purposes of obtaining the benefit of the filing date of the parent application was found where

*** the subgeneric claims of the continuation-in-part applications (1) are completely within the scope of the parent case generic disclosure and (2) read on at least one species disclosed in a working example of the parent application.

As we have previously stated, the one method for which reference was made to the Tiers application may very well be sufficient to teach one skilled in the art how to make the claimed esters. Certainly the claims, inasmuch as esters wherein m is 3-12 are also covered, read upon a species disclosed in a working example of the parent application. However, the first part of the *Risse* requirement has not been met. The esters of claim 7 on appeal are not completely within the scope of the parent case generic disclosure. Appellant's argument that ω -perfluoroalkyl-substituted acrylate esters is the described genus and that those wherein m is 2-12 is the subgenus is untenable. As we have previously noted, the esters wherein m is 3-12 comprise the only genus described in full, clear, concise, and exact terms as required by the statute. Some esters wherein m is 2-12 clearly fall outside the class of esters described in the parent application.

Therefore, we *affirm* the decision of the board.

Patent Office Board of Appeals

Ex parte PALM

Patent issued Dec. 29, 1970

Opinions dated Oct. 22 and Dec. 22, 1969

PATENTS

1. Patentability — Aggregation or combination — Improving old element (§51.155)

Fact that references disclose mature

154 USPQ

Decisions of the United States Courts and of the

United States Patent Office in

Patent, Trademark, and Copyright Cases

54 CCPA 1495

Court of Customs and Patent Appeals

In re RISSE, HORLEIN, AND WIRTH;
In re HORLEIN, WIRTH, AND RISSE

Appl. Nos. 7574, 7577

Decided June 15, 1967

PATENTS

1. Specification — Sufficiency of disclosure (§ 62.7)

Subgeneric claim of continuation-in-part application is entitled to benefit of filing date of parent application where claim is completely within scope of parent application generic disclosure and reads on at least one species disclosed in working example of parent application.

2. Interference—Reduction to practice—In general (§ 41.751)

Interference—Reduction to practice—Constructive reduction (§ 41.755)

Foreign inventors who made their inventions abroad are precluded by 35 U.S.C. 104 from establishing their dates of invention by actual reduction to practice and are restricted to their effective filing dates for proving dates of invention by constructive reductions to practice.

3. Patentability — Anticipation—Involving interference (§ 51.213)

There is no reasonable basis for contention that award or concession of priority necessarily makes complete disclosure of winning party's application available as prior art, either by itself or in combination with other art, against losing party's application.

4. Estoppel — Involving interference (§ 35.20)

Patentability — Anticipation — Involving interference (§ 51.213)

Disclosures in application of winning

interference party may be used against claims in losing party's application under separate principles of (1) interference estoppel and (2) statutory prior art under 35 U.S.C. 102 (g) and 103.

5. Estoppel — Involving interference (§ 35.20)

Under judicial doctrine of interference estoppel, not all disclosures of winning party's application can be used against losing party's claims, but only those disclosures which are clearly common to both applications in interference.

6. Patentability — Anticipation — Involving interference (§ 51.213)

Patentability — Invention — In general (§ 51.501)

Prior art under 35 U.S.C. 102 includes prior invention under section 102(g); at a minimum, prior invention under section 102(g) includes subject matter of interference counts, which may be used as evidence of prior art under section 103; while section 102(g) includes subject matter of counts, priority of invention as to which was actually determined adversely to losing party, it does not include common subject matter outside scope of interference counts, priority of invention as to which subject matter might have been determined in interference proceeding.

7. Estoppel — Involving interference (§ 35.20)

Patentability — Anticipation — Involving interference (§ 51.213)

1952 Patent Act did not resolve judicial conflict in relation to doctrine of interference estoppel; 35 U.S.C. 102(g) merely retains rules of law governing determination of priority of invention developed by judicial and administrative decisions in interference proceedings.

8. Estoppel — Involving interference (§ 35.20)

Patentability — Anticipation—Involving interference (§ 51.213)

Interference estoppel and prior art are separate and distinct matters which should not be confused; therefore, claims which winning party could not make, for lack of disclosure, cannot be denied to loser on ground of interference estoppel, if they distinguish patentably from counts; distinction which should be borne in mind is that, with regard to interference estoppel, losing party is only estopped to obtain claims which read directly on disclosures of subject matter clearly common to both winning party's and losing party's applications; but that, with regard to prior art (including prior invention), losing party cannot obtain claims to subject matter which is either barred under 35 U.S.C. 102(g), or rendered obvious under section 103, by invention defined in interference counts.

9. Estoppel — Involving interference (§ 35.20)

Although priority of invention as to certain species was not determined in interference, priority might have been so determined since species represents commonly disclosed subject matter; thus, losing party is estopped to obtain claim reading directly on species, regardless of whether species is prior invention of winning party in terms of 35 U.S.C. 102(g).

10. Estoppel — Involving interference (§ 35.20)

Patentability — Anticipation — Involving interference (§ 51.213)

Fact that applicants (losing interference party) are estopped by interference to claim patentable subject matter clearly common to both their application and that of winning party does not necessarily make common disclosures of one subgeneric invention "prior art" under 35 U.S.C. 102(g) and 103 as to a different subgeneric invention even though both subgeneric inventions are embraced within generic concept disclosed and claimed in applicants' application; therefore, Patent Office may not use commonly disclosed species as evidence of prior art unless and until that compound becomes available statutory prior art, as for example by issuance of patent on winning party's application.

11. Patentability — Anticipation — Patents — On pending applications (§ 51.2219)

Patent issued on application

tion is prior art as of application's filing date.

12. Patentability — Anticipation — Involving interference (§ 51.213)

Interference count as to which applicants conceded priority of invention to opponent is admissible evidence of prior art under 35 U.S.C. 102(g) and 103.

13. Patentability—Composition of matter (§ 51.30)

Claimed compounds which are three to five times better than prior art compound do not differ therefrom only in degree but differ in kind; there is no evidence that such improvement would have been expected by one of ordinary skill in the art.

14. Patentability — Anticipation — Involving interference (§ 51.213)

In so far as In re Bicknell, 58 USPQ 553, and In re Boileau, 78 USPQ 146, hold that all commonly disclosed subject matter is prior art against losing interference party's claims, they are overruled; although all subject matter which is clearly common to applications of winning and losing parties may be used for purposes of interference estoppel rejection against losing party's claims, extent to which commonly disclosed subject matter may be used as available evidence of prior art under 35 U.S.C. 103 depends on whether common subject matter relied on meets section 102; this depends on facts of the particular case; general rules, e.g., that all commonly disclosed subject matter is prior art against losing party's claims, are not to be blindly applied in particular cases in which facts may differ from controlling facts in precedents wherein such generalities are expressed.

Particular patents—Derivatives

Risse, Horlein, and Wirth, Phenothiazine Derivatives, claim 44 of application allowed; claim 47 refused.

Appeals from Board of Appeals of the Patent Office.

Application for patent of Klaus Heinz Risse, Ulrich Horlein, and Wolfgang Wirth, Serial No. 26,707, filed May 4, 1960, Patent Office Group 120. From decision rejecting claims 44 and 47, applicants appeal. Affirmed as to claim 47; reversed as to claim 44.

Application for patent of Ulrich Horlein, Wolfgang Wirth, and Klaus Heinz Risse, Serial No. 608,170, filed Sept. 5, 1956, Patent Office Group 120. From decision rejecting claim 9, applicants appeal. Appeal dismissed.

Smith, Judge, concurring with opinion.

OLD S
for al
JOSEPH
of col
ents.

Before :
MARTI
ate J.
ALMO

These
decisions:
Appeals
claim 9
serial 2
1950, an
ation-in-
707, file
"Phenot

The i
phenoth
gungtion
ating at
therapeu
reducing
emetics.

Claim
broadest
and illu
of the co

9. A



in whi
and Y

Appell
that clai
tially the
in claim
case and
ments to
applicati
only for
claims, w

44.
azyl-N-
in whi
lected
propior
alkylem
atoms.
47 2

rior art as of application's fil-

stability — Anticipation — In-

ving interference (§ 51.213)
 amount count as to which appli-
 cation's priority of invention to
 is admissible evidence of prior
 art, 35 U.S.C. 102(g) and 103.

stability — Composition of mat-
 ter (§ 51.30)

1 compounds which are three
 times better than prior art com-
 pounds not differ therefrom only in
 at differ in kind; there is no
 that such improvement would
 be expected by one of ordinary
 skill in the art.

stability — Anticipation — In-
 terference (§ 51.213)

as in *In re Bicknell*, 58 USPQ
 146, 78 USPQ 146, all commonly disclosed sub-
 ject matter is prior art against losing
 party's claims, they are
 ; although all subject matter
 clearly common to applications
 and losing parties may be
 purposes of interference estop-
 plement against losing party's
 claim to which commonly dis-
 cussed matter may be used as
 evidence of prior art under
 103 depends on whether com-
 mon matter relied on meets sec-
 tion 103 depends on facts of the
 case; general rules, e.g., that
 only disclosed subject matter
 art against losing party's
 claims is not to be blindly applied in
 cases in which facts may dif-
 ferencing facts in precedents
 claim generalities are expressed.

lar patents — Derivatives

Horlein, and Wirth, Phenothia-
 zines, claim 44 of application
 claim 47 refused.

from Board of Appeals of
 Office.

tion for patent of Klaus Heinz-
 rich Horlein, and Wolfgang
 al No. 26,707, filed May 4,
 1956, Office Group 120. From de-
 ciding claims 44 and 47, appli-
 cation. Affirmed as to claim 47;
 reversed as to claim 44.

for patent of Ulrich Hor-
 lein, and Wolfgang Heinz-
 rich Wirth, and Klaus Heinz-
 rich al No. 608,170, filed Septem-
 ber 1956, Office Group 120. From
 deciding claim 9, applicants ap-
 pealed.

OLD SPRUNG, both of New York, N. Y.,
 for appellants.

JOSEPH SCHMIDT (RAYMOND E. MARTIN
 of counsel) for Commissioner of Pat-
 ents.

Before WORLEY, Chief Judge, and RICH,
 MARTIN, SMITH, and ALMOND, Associ-
 ate Judges.

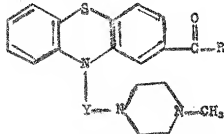
ALMOND, Judge.

These are consolidated appeals from
 decisions of the Patent Office Board of
 Appeals affirming the rejections of
 claim 9 of appellants' parent application
 serial No. 608,170, filed September 5,
 1956, and claims 44 and 47 of contin-
 uation-in-part application serial No. 26,
 707, filed May 4, 1960, both entitled
 "Phenothiazine Derivatives."

The invention relates to novel acyl
 phenothiazines which possess excellent
 ganglion-blocking, hypotensive, potenti-
 ating and spasmolytic activity and are
 therapeutically useful as blood pressure
 reducing agents, sedatives, and anti-
 emetics.

Claim 9 of the parent case is the
 broadest of the three claims on appeal
 and illustrates the structural formulae
 of the compounds:

9. A compound having the formula



in which R is a lower alkyl radical
 and Y is a lower alkylene radical.

Appellants acknowledge in their brief
 that claim 9 is directed to "substan-
 tially the same invention as that claimed
 in claim 44" of the continuation-in-part
 case and accordingly limit their argu-
 ments to claims 44 and 47 of the second
 application, relying upon the parent case
 only for support for the two latter
 claims, which read as follows:

44. A 3-acyl-10-(N'-methyl-piper-
 azyl-N-lower alkylene)-phenothiazine
 in which said acyl is a member se-
 lected from the group consisting of
 propionyl and butyryl and said lower
 alkylene contains 2 to 3 carbon

The references relied upon are:

Robinson et al. 2,590,125 March 25,
 1952

Cusick 2,650,919 September 1, 1953

Sherlock 2,385,554 May 23, 1961
 (filed Sept. 21, 1956)

Belgian Patent 552,256 November 14,
 1956

Application of Schmitt (winning
 party of Interference No. 89,699 in
 which appellants conceded priority in
 parent case serial No. 608,170).

The effective reference dates of Sher-
 lock and the Belgian patent are subse-
 quent to the September 5, 1956 filing
 date of appellants' parent case. Conse-
 quently, the first issue is whether the
 two claims of appellants' continuation-
 in-part case are entitled under 35 U.S.C.
 120 to the benefit of the filing date of
 their expiring parent application,
 which in turn depends on whether the
 invention of claims 44 and 47 is suffi-
 ciently disclosed and described in the
 parent case in the manner required by
 35 U.S.C. 112, so as to enable an ordi-
 narily skilled pharmaceutical chemist to
 make and use the claimed compounds.

With regard to this first issue, the
 board stated:

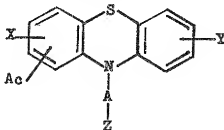
As the examiner explains, appel-
 lants are not entitled to the date of
 their application *with respect to the*
claimed subject matter of the present
application since the parent applica-
 tion describes only one compound fall-
 ing within the subgenus of claim 44
 and, although it contains broad, in-
 clusive terminology, provides no spe-
 cific disclosure of the subgenus of
 claim 44 or the compound of claim 47.
 We cannot see that *In re Grimme*
 et al., 47 CCPA 785, 1960 C.D. 123,
 754 O.G. 6, 274 F.2d 949, 124 USPQ
 499, which appellants invoke, aids
 their cause. With respect to claim 44
 the situation appears to be more
 nearly that of *Watson v. Bersworth*
 et al., 102 U.S. App. D.C. 187, 1958
 C.D. 46, 727 O.G. 445, 251 F.2d 986,
 116 USPQ 73; *In re Shokal et al.*, 44
 CCPA 851, 1957 C.D. 234, 718 O.G.
 445, 242 F.2d 771, 113 USPQ 283, or
In re Fried, 50 CCPA 954, 1963 C.D.
 248, 793 O.G. 7, 312 F.2d 930, 136
 USPQ 429, since there is no definite
 support for the subgenus. * * *

[Emphasis board's.]

We disagree with the board on this
 issue. The parent application not only
 contains a working example for one
 compound within the subgenus of claim

ample 8, but also contains the following generic disclosure which is inclusive of the subgenus of claim 44:

The novel phenothiazine derivatives in accordance with the invention have the general formula



in which Ac represents an acyl radical, such as a lower, straight or branched chain alkyl acyl radical having, for example, between 2 and 4 carbon atoms, or an aryl acyl radical, as, for example, the benzoyl radical; X and Y represent hydrogen atoms or lower molecular weight mono-valent substituents, such as lower alkyl or alkoxy radicals or halogen atoms, as, for example, methyl radicals or chlorine atoms; A represents a lower straight or branched chain alkylene radical, such as the methylene, ethylene or propylene radical; Z is a lower dialkylamino radical or a heterocyclic radical, such as a piperidino-, pyrrolidino-, morpholino- or piperazino-radical, which may possibly be substituted. [Emphasis ours.]

The emphasized portions of the above generic disclosure correspond to the subgenus of claim 44. The parent case also states that "the acyl radical is * * * preferably positioned at the 3-position" and that "when the X and Y represent hydrogen, the homocyclic rings are, of course, unsubstituted." The parent case also includes 27 working examples, among which are twelve directed to 3-propionyl phenothiazines within the generically disclosed invention, two directed to 3-butyl compounds, eight in which the lower alkylene bridge (A or Y) is propylene (including five wherein the point of attachment to the terminal Z group is the γ -carbon and three for the β -carbon), and five wherein the bridge between the 10- or N- position of the phenothiazine nucleus and the terminal Z group is ethylene.

In view of the circumstances of this particular case, we believe our Grimme decision, wherein one working example and broad generic disclosure was held to be adequate support under 35 U.S.C.

112 for a claim to a subgenus not expressly and specifically disclosed as such, is more apposite than the cases cited by the board and is properly applicable here. We recognize that the examiner had indicated in the Grimme case that a generic claim to all the compounds would have been allowable in the parent case, if it had been presented, while such is not the case here. On the other hand, such a generic claim had not actually been allowed in Grimme's parent application and the subgeneric claim which we held to be properly supported read on considerably more species than claim 44 here, which the solicitor views as inclusive of only 18 specific acylphenothiazines.

The three cases cited by the board may readily be distinguished. *Watson v. Bersworth*, 251 F.2d 898, 116 USPQ 79, represents a two to two split among judges of the District of Columbia courts on the issue of the sufficiency of generic disclosure in a parent application to support subgeneric claims in a continuation-in-part application. Two of the three opinions in the case, those of the trial judge, 159 F.Supp. 12, 116 USPQ 87, and the dissenting circuit judge, 251 F.2d at 901, 116 USPQ at 80, disagree with the position of the Patent Office. Furthermore, the facts of the case as set forth in the dissenting circuit judge's opinion, the only one citing section 120, are quite different from those of the cases on appeal here, in that not one of the species embraced within the two subgeneric claims was specifically disclosed in the first (parent) case. The first specific disclosure of such subject matter was in an intermediate continuation-in-part application, identified as Case B in the dissent.²

The Fried decision cited by the board is similar to *Watson v. Bersworth* in that the parent case likewise did not specifically disclose a single compound within the scope of the claimed subgenus. In this respect, the court said:

* * * it is clear, as pointed out by the examiner, that there is no disclosure of a specific method of preparation of the specific compounds claimed here and, as pointed out by

¹ The two variables in Grimme's subgeneric claim 1 on appeal were defined as follows: "R₁ is a radical containing not in excess of 10 carbon atoms and selected from the group consisting of hydrogen, alkyl, and phenylalkyl radicals; and A is a saturated aliphatic hydrocarbon radical having 2 to 5 carbon atoms." 47 CCPA at 786, 274 F.2d at 909, 124 USPQ at 890.

² 251 F.2d at 906, 116 USPQ at 85.

be demonstrated by reference to the facts of record in this case. Both Schmitt and appellants are foreign inventors who filed their first patent applications abroad. Schmitt's application serial No. 675,096 was actually filed in this country March 30, 1956, and a priority date of June 30, 1955 for a counterpart French application was claimed pursuant to 35 U.S.C. 119. Appellants' parent application was actually filed in this country September 5, 1956, after a German application was filed September 7, 1955, nearly one year earlier. Thus, each of Schmitt's foreign and actual United States filing dates is prior to the corresponding filing date of appellants, and we infer that this adverse position as to filing dates⁶ was the basis for appellants' concession of priority as to the compound of the interference count, 3-acetyl-10-(7-dimethylaminopropyl)-phenothiazine, also known as acetyl-promazine.

However, neither Schmitt's nor appellants' foreign applications are of record in this case. It may well be, for all we know, that Schmitt's French application contained only one example, directed to the species of the count of the interference, and that the remaining disclosure of Schmitt's United States application, which is of record, was new matter disclosed for the first time when Schmitt filed in this country, in which event Schmitt's American application would be entitled to the benefit of his French filing date only for the compound of the interference count. It may also be that appellants' United States parent and German applications are substantially identical, in which case their date of invention would be their German filing date of September 7, 1955, which precedes Schmitt's actual United States filing date of March 30, 1956 by nearly seven months.

The significance of the above is that the Patent Office tribunals are not, at least primarily, rejecting appellants' claims as unpatentable over the interference count, but rather are primarily rejecting them as unpatentable over a compound, not directly involved in the interference, which is common subject matter to both applications, namely, 3-propionyl-10-(7-dimethylaminopropyl)-phenothiazine, further in view of the

⁶ [2] Schmitt and appellants, all foreign inventors who made their inventions abroad, would be precluded by 35 U.S.C. 104 from establishing their dates of invention by actual reduction to practice, and would be restricted to their effective filing dates for proving dates of invention by

Cusick and Robinson patents. These secondary references establish, according to the board, the prima facie plausibility of dialkylamino and heterocyclic aliphatic amino (including 4-methylpiperazinyl) radicals as terminal tertiary-amino groups connected to the 10- or N- position of the phenothiazine nucleus by means of a lower alkylene bridge. This art-recognized equivalence is also acknowledged in appellants' parent application, wherein heterocyclic radicals and lower dialkylamino radicals are equated as terminal group Z in the generic structural formula.

[5] We see no reasonable basis for a contention that an award or concession of priority necessarily makes the complete disclosure of the winning party's application available as prior art, either by itself or in combination with other art, against the losing party's application. As noted above, appellants' parent application may well be prior as to everything in Schmitt's United States application, except the count of the interference, as to which appellants conceded priority. We take note of the solicitor's reliance upon *In re Gregg*, 44 CCPA 904, 244 F.2d 316, 113 USPQ 526. However, in that case a patent had actually issued on the winning party's application, so that the complete disclosure of the patent was in fact available prior art under 35 U.S.C. 102(e) and 103 as of the application filing date. See *In re Taub*, 52 CCPA 1675, 343 F.2d 556, 146 USPQ 384, 389. In the present case, the record does not reveal that a patent has issued on the Schmitt application.

Although the Gregg case holding does not support the Patent Office position that the application disclosures of the winning party in an interference proceeding are available prior art under 35 U.S.C. 102(g) and 103 against the losing party's claims, we must admit that there are decisions of this court from which that conclusion could be drawn. For example, this court held in *In re Bicknell*, 30 CCPA 1250, 1253, 136 F.2d 1016, 1018, 58 USPQ 553, 556, that "as far as appellants are concerned the application of Jorgensen and that of Jocke [the winning interference parties] are prior art."

Also, in *In re Boileau*, 35 CCPA 1248, 168 F.2d 753, 78 USPQ 146, a patent had already issued on the winning interference party's application, but the filing date of the application was not early enough for the complete disclosure of the patent to be available as a reference under the rule of *Alexander Milburn Co. v. Davis-Bourneville Co.*, 270 U.S. 340, now entitled *In re Milburn*.

paten
as to
closed
party
well s
and (C
Boileu
hold i
win hi
again;
combi
nate
able c
tion."

Thu
court's
the P.
Office
comm
conce
tion is
and
claims
law s
of w
matte

[4]
102(c)
case,
applic
again
separ
estopp
under
statem
interf
cision
CCPA
140 U
made

*
cles
unde
terfo
tion
nigh
min
[3]
esto
part
test
natu
his
his
cted

[5]
interf
all of
could
Only t
comin
ference

and Robinson patents. These secondary references establish, according to the prima facie equivalence of alkylamino and heterocyclic alkylamino (including 4-methylpiperidyl radicals as terminal tertiaryamino groups connected to the 10- or 11-position of the phenothiazine ring) by means of a lower alkylene group. This art-recognized equivalence is acknowledged in appellants' participation, wherein heterocyclic and lower dialkylamino radicals are listed as terminal group Z in the structural formula.

We see no reasonable basis for contention that an award or concession of priority necessarily makes the disclosure of the winning application available as prior art, by itself or in combination with art against the losing party's application. As noted above, appellants' application may well be prior art in Schmitt's United States patent, except the count of the invention, as to which appellants concede priority. We take note of the reliance upon *In re Gregg*, 44 USPQ 904, 244 F.2d 316, 118 USPQ 904, where in that case a patent had been issued on the winning party's invention, so that the complete disclosure of the patent was in fact available prior art under 35 U.S.C. 102(e) and of the application filing date. See also, 52 CCPA 1675, 348 F.2d 556, 138 USPQ 384, 389. In the present case, the court does not reveal that a patent was issued on the Schmitt application. Although the *Gregg* case holding supports the Patent Office position at the application disclosures of the winning party in an interference proceeding are available prior art under 35 U.S.C. 102(g) and 103 against the party's claims, we must admit that such decisions of this court which that conclusion could be reached.

For example, this court held in *In re Risse*, 35 CCPA 1248, 154 USPQ 146, a patent issued on the winning party's application, but the disclosure of the application was not sufficient for the complete disclosure of the invention as a reference prior art."

patent to be a proper prior art reference as to all subject matter commonly disclosed in both the patent and losing party Boileau's application, citing *Bicknell* as authoritative precedent. *Rivise and Caesar*, in *Interference Law and Practice*, Vol. IV, p. 2922 (1948), cite Boileau as exemplary of cases "which hold in effect that the application of the winning party constitutes prior art against the losing party and may be combined with other prior art to anticipate [render obvious] otherwise allowable claims in the losing party's application."

Thus there is some support in this court's decisions, prior to enactment of the Patent Act of 1952, for the Patent Office position that, at least insofar as commonly disclosed subject matter is concerned, the winning party's application is prior art under 35 U.S.C. 102(g) and 103 against the losing party's claims. Aside from the matter of case law support, however, we think the issue of whether the position is correct or not requires searching investigation.

[4] Although the effect of section 102(e) is not a factor to consider in this case, unlike *Gregg*, supra, the Schmitt application disclosures may still be used against appellants' claims under the separate principles of (1) interference estoppel and (2) statutory prior art under 35 U.S.C. 102(g) and 103. A good statement of the judicial doctrine of interference estoppel is found in the decision of this court in *In re Yale*, 52 CCPA 1668, 1674, 347 F.2d 995, 1001, 146 USPQ 400, 404, wherein reference is made to:

"* * * the well established principle of estoppel that an interference settles not only the rights of the parties under the issues or counts of the interference but also settles every question of the rights to any claim which might have been presented and determined in the interference proceeding. [Cases cited.] * * * the doctrine of estoppel has been applied where a party has neglected or refused to contest priority of patentable subject matter which is clearly common to his application and the application of his opponent in interference [cases cited, emphasis in original] * * *"

[5] Under the judicial doctrine of interference estoppel, it is clear that not all of the Schmitt application disclosures could be used against appellants' claims. Only those disclosures which are clearly

and the two appealed claims of appellants' continuation-in-part application are strictly limited to such compounds, it should be evident that appellants are not estopped to present and obtain these claims, which do not by any stretch of the imagination read on the application disclosures of Schmitt, their interference adversary.

[6] Proceeding now to the matter of statutory prior art, we think it is well settled that prior art under 35 U.S.C. 103 includes prior invention under 35 U.S.C. 102(g). See, e.g., *In re Yale*, supra. Section 102(g) reads in pertinent part as follows:

A person shall be entitled to a patent unless—

(g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it * * *.

At a minimum, prior invention under section 102(g) includes the subject matter of the interference counts, which may be used as evidence of prior art under section 103. As this court stated in *In re Cole*, 23 CCPA 1057, 1063, 82 F.2d 406, 409, 29 USPQ 187, 141:

We therefore hold, in harmony with our decisions hereinbefore cited, that in order to warrant the allowance of the claims before us the claims must be inventively different from said interference counts; or, in other words, the specific details or limitations in the claims before us not found in said interference counts must, when combined with the structure embraced in said counts, involve [patentable] invention over said counts.

While it is clear that 35 U.S.C. 102(g) includes the subject matter of the interference counts, priority of invention as to which was actually determined adversely to the losing party, it is not clear whether or not section 102(g) includes the subject matter clearly common to the applications of both interference parties, but outside the scope of the interference counts, priority of invention as to which subject matter might have been determined in the interference proceeding. One thing is clear, however. If a commonly disclosed subject matter is treated as conclusive evidence of prior invention under section 102(g), then any distinction between the judicial doctrine of interference estoppel

would automatically be regarded as available prior art under 35 U.S.C. 103 against the losing party in an interference.

Upon review of prior decisions of this court, notably, Bicknell and Boleau, *supra*, we find that these two separate and distinct matters have often been confusingly interrelated. This confusion has been judicially criticized by the Court of Appeals for the District of Columbia Circuit in *Ethyl Gasoline Corp. v. Coe*, 139 F.2d 872, 873-4, 59 USPQ 465, 457 (1943), which pointed out that "[t]he confusion, between an estoppel and the condition of the prior art, has been unfortunate and misleading."

Prior to enactment of the Patent Act of 1952, various commentators likewise recognized the existence of a judicial conflict concerning the nature and scope of the doctrine of interference estoppel, which bears on the issue with which we are presently concerned, namely whether the commonly disclosed subject matter, as to which a recognized estoppel exists, may be used as prior art under 35 U.S.C. 103 against the losing party's claims. McCrady, in his book *Patent Office Practice*, Second Edition, 1946, pp. 161-3, comments as follows:

Because the doctrine of interference estoppel as applied by the Court of Customs and Patent Appeals and the [Patent] Office is not based upon any definite provision of the statutes or upon any legal doctrine recognized in courts of general jurisdiction [including the Court of Appeals, D.C.], the scope of the doctrine, and the criteria by which its occurrence is recognized, have varied considerably as succeeding decisions were rendered. * * * The doctrine is peculiar to practice in the Patent Office, the Court of Customs and Patent Appeals, and (in modified form) the courts of the District of Columbia; it has never been recognized by other courts.

* * * decisions [of the Court of Appeals for the District of Columbia] have applied the interference estoppel doctrine in a more restricted form than have the Patent Office and [this court]. * * *

Conformably with the rule of bar by judgment, the District of Columbia courts hold that no interference estoppel arises where the claims in question could not have been made counts of the existing interference * * *. On this point, the decisions of the Dis-

trict with those of the Court of Customs and Patent Appeals. [Emphasis ours.]

[7] When Congress enacted the Patent Act of 1952, no resolution was made of this recognized judicial conflict in relation to the doctrine of interference estoppel. Paragraph (g) of 35 U.S.C. 102 merely retains the rules of law governing the determination of priority of invention developed by judicial and administrative decisions in interference proceedings. Reviser's Note, 35 U.S.C.A. 102 (2); Federico, "Commentary on the New Patent Act," 35 U.S.C.A. p. 19 (1954). Since Congress did not choose to resolve the conflict by statute, this leaves the courts free to attempt harmonization of conflicting precedents, insofar as desired. We are persuaded to adopt the more liberal view of the Court of Appeals for the District of Columbia.

[8] Circuit that interference estoppel and prior art are separate and distinct matters which should not be confused. The result is adoption of the following position stated by McCrady, *supra*, p. 164:

But claims which the winning party could not make, for lack of disclosure, cannot be denied to the loser on the ground of interference estoppel, if they distinguish patentably from the counts. * * *

The distinction which should be borne in mind is that, with regard to interference estoppel, the losing party is only estopped to obtain claims which read directly on disclosures of subject matter clearly common to both the winning party's application and that of the losing party; but that, with regard to prior art (including prior invention), the losing party cannot obtain claims to subject matter which is *either* barred under 35 U.S.C. 102(g), or rendered *obvious* under 35 U.S.C. 103, by the invention defined in the interference counts.

[9] Applying these principles to the case at bar, we note that an interference estoppel exists as to the species of example 3 of the Schmitt application, 3-propionyl - N-(7-dimethylamino-propyl)-phenothiazine, since this compound is also disclosed in examples 2, 18, and 27 of appellants' parent application. Although priority of invention as to this species was not actually determined in the interference, priority *might* have been so determined, since it represents commonly disclosed subject matter. Thus appellants are estopped to obtain a claim which reads directly on this dimethylamino species, *regardless* of whether the compound is the prior in-

of 35
Eitzen,
USPQ 5

Appel
of the c
to that
pealed c
have m
the
8, or a
live dis
ter.⁷
Altho
termina
of the s
ence, th
mination
record,
is outst
interfer
art unde

[10]
stress h
appellar
ence to
which i
parent
namely
of the d
sarily r
one sul
under 3
differen
the ph
methyl
now cla
invento
eric co
spellar
Under
that the
use the
ample 8
art aga
unless s
availabl

[11]
ent on
would n
Schmitt
country.

[12]
Office
of the
(7-dime
zine, pr
was cor
we thin

⁷ Cf. 1
146 F.2d
the Cou
Columbi
estoppel
pealed)
applicat
party.]

154 USPQ

those of the Court of Customs and Patent Appeals. [Emphasis

n Congress enacted the Patent Act of 1952, no resolution was made of the doctrine of interference. Paragraph (g) of 35 U.S.C. 102 ins the rules of law governing the determination of priority of inventions by judicial and administrative actions in interference proceedings. See, e.g., *Ex parte* [redacted], 35 U.S.C.A. 102 (1954). It is not proper to rely on precedents, insofar as they are not binding, to adopt the view of the Court of the District of Columbia that interference estoppel is a separate and distinct doctrine which should not be confused with the doctrine of estoppel by adoption of the following language from *McCrady*, supra, p.

which the winning party makes, for lack of disclosure, denied to the loser on the interference estoppel, if it is patentably from the

action which should be borne in mind with regard to interference, the losing party is only entitled to obtain claims which read disclosures of subject matter common to both the winning and losing parties and that of the loser that, with regard to prior art, the losing party is not entitled to obtain claims to subject matter which is either barred under 35 U.S.C. 102(g), or rendered obvious by the invention disclosed in the interference counts.

ing these principles to the case at hand, we note that the interference counts as to the species of estoppel in the Schmitt application, 3-N-(dimethylamino)propylamine, since this compound is disclosed in examples 2, 18, and 19 of the parent application. All rights of invention as to this compound are not actually determined in the interference proceedings, since it represents disclosed subject matter. The parties are estopped to obtain claims which read directly on this compound, regardless of whether the compound is the prior in-

154 USPQ

In re Risse, Horlein, and Wirth

of 35 U.S.C. 102(g). See *Dirkes v. Eitzen*, 26 CCPA 1198, 103 F.2d 520, 41 USPQ 546.

Appellants recognize the applicability of the doctrine of interference estoppel to that compound. None of their appealed claims, which Schmitt could not have made for lack of disclosure, reads on the compound of Schmitt's example 8, or any other phenothiazine derivative disclosed by Schmitt for that matter.

Although there might have been a determination as to priority of invention of the aforesaid species in the interference, there was in fact no such determination, and the Patent Office, on this record, cannot use this compound, which is outside the scope of the specific interference count, as evidence of prior art under 35 U.S.C. 102(g) and 103.

[10] The important thing which we stress here is that the mere fact that appellants are estopped by the interference to claim patentable subject matter which is clearly common to both their parent application and that of Schmitt, namely certain phenothiazine derivatives of the dialkylamino type, does not necessarily make such common disclosures of one subgeneric invention "prior art" under 35 U.S.C. 102(g) and 103 as to a different subgeneric invention, namely the phenothiazine derivatives of the methylpiperazyl type which appellants now claim, even though both subgeneric inventions are embraced within the generic concept disclosed and claimed in appellants' parent application.

Under the circumstances, we think that the Patent Office may not properly use the dimethylamino species of example 8 of Schmitt as evidence of prior art against appellants' present claims, unless and until that compound becomes available statutory prior art, as for example [11] ample by the issuance of a patent on the Schmitt application, which would make this species prior art as of Schmitt's application filing date in this country. 35 U.S.C. 102(e), 103.

[12] To the extent that the Patent Office secondarily relies on the species of the interference count, 3-acetyl-10-(7-dimethylamino)propyl-1-phenothiazine, priority of invention as to which was conceded to Schmitt by appellants, we think the Wirth affidavit of record

is adequate to establish patentability of the presently claimed phenothiazine derivatives of the methylpiperazyl type over this dimethylamino species, which is admissible evidence of prior art under 35 U.S.C. 102(g) and 103. In re Yale, supra. Smith v. Watson, 218 F.2d 863, 104 USPQ 160 (D.C. Cir. 1955).

The board has expressed three objections to this affidavit. One of these, that the properties compared were not disclosed in appellants' continuation-in-part application, is clearly erroneous and has been withdrawn by the solicitor's brief. A second objection, that the "prior art" compound tested by appellants, the 3-acetyl species of the interference count, is "obviously not the closest in structure to the compounds claimed," is not well taken since the 3-propionyl species commonly disclosed in both applications involved in the interference is not legally "prior art" as to appellants' claims on the record of this case, for reasons discussed in detail above.

[13] The third objection to the Wirth affidavit, that the results differ only in degree but not in kind, is unfounded in our opinion. We disagree particularly with the examiner's view that the results proving two of the claimed compounds to possess a circulatory regulation capacity at least three to five times better than that of the 3-acetyl compound defined by the interference count show a "difference of degree only." There is no evidence of record showing that such improvement would have been expected by one of ordinary skill in this art. The claimed compounds might have been three to five times worse than the prior art compound. Instead, they are three to five times better. See In re Wagner, 54 CCPA 1031, 371 F.2d 877, 152 USPQ 552.

[14] Insofar as the *Ricknell* and *Bolleau* cases, supra, hold that "all" commonly disclosed subject matter is "prior art" against the losing interference party's claims, those cases are expressly overruled, as they are inconsistent with the views expressed herein as to the entirely separate and distinct nature of the judicial doctrine of interference estoppel and the statutory prior art under 35 U.S.C. 103, the latter including prior invention under 35 U.S.C. 102(g). Although "all" subject matter which is clearly common to the applications of the winning and losing interference parties may be used for purposes of an interference estoppel rejection against the losing party's claims, the extent to which this commonly disclosed subject matter may be used as available evidence of the "prior art" under section 103 depends on whether

⁷ Cf. *United States Rubber Co. v. Cooe*, 145 F.2d 315, 64 USPQ 100, 101, wherein the Court of Appeals for the District of Columbia Circuit affirmed an interference estoppel rejection since "all the [appealed] claims are readable on the * * * application [of the winning interference party] * * *."

the common subject matter relied on meets one or more of the paragraphs of 35 U.S.C. 102. This, of course, will in turn depend on the facts and circumstances of a particular case. General rules, e.g. that "all" commonly disclosed subject matter is "prior art" against the losing party's claims, in *re Boileau*, supra, are to be neither trusted nor blindly applied in particular cases in which the facts may well differ materially from the controlling facts in precedents wherein such generalities are expressed.

For the reasons stated above, the decision of the board in PA 7574 is reversed as to subgeneric claim 44 and affirmed as to species claim 47, and the appeal in PA 7577 is dismissed as moot.

WORLEY, Chief Judge, concurs in the result.

MARTIN, Judge, participated in the hearing of this case but died before a decision was reached.

SMITH, Judge, concurring.

The record shows that the Board of Appeals here consisted of an examiner-in-chief and two acting examiners-in-chief. Appellants do not challenge the legality of that board. For the reasons expressed in my dissenting opinion in *In re Wiechert*, 54 CCPA 957, F.2d 927, 152 USPQ 247, the decision of such a board in my view is a legal nullity. However, I must accept the majority's view on this issue in the *Wiechert* case, i.e., the legality of the board is not an issue here. I therefore participate in the merits of this appeal and in so doing, agree with the conclusion of the majority.

54 CCPA 1466

Court of Customs and Patent Appeals

In re KALM

Appl. No. 7698 Decided June 15, 1967

PATENTS

I. Patentability — Anticipation — In general (§ 51.201)

Rejection under 35 U.S.C. 102(e) for anticipation necessarily implies that invention sought to be patented is not new, i.e., that there are no differences between what is claimed and what is disclosed in prior art.

2. Patentability—Anticipation — In general (§ 51.201)

Patentability — Invention — In general (§ 51.501)

Description in reference which is insufficient as a matter of law to render composition of matter obvious (35 U.S.C. 103) to one of ordinary skill in the art would a fortiori be insufficient to "describe" the composition as that term is used in section 102(e), a complete description being but the ultimate or epitome of obviousness.

3. Patentability — Composition of matter (§ 51.30)

Where reference patent imputes particular characteristics to a readily prepared, specifically named and identified compound or composition, a party seeking to claim same compound or composition must prove that patent description was erroneous and that what the patent appears to expressly describe never actually existed.

4. Words and phrases (§ 70.)

When one speaks of a "genus" in chemical arts, one ordinarily speaks of a group of compounds closely related both in structure and properties.

5. Patentability — Composition of matter (§ 51.30)

While it is not necessary that reference disclose every property or attribute of a composition of matter to be a valid anticipation, applicant has found properties for his claimed compounds which are totally incompatible and inconsistent with, not merely complementary or in addition to, those attributed by patentee to his compounds; patentee does not disclose compounds within scope of applicant's claims.

Particular patents—Morpholine Derivatives

Kalm, 3,4-Dialkyl - 2-Cycloalkylmorpholines and Congeners, claims 1 to 3 of application allowed.

Appeal from Board of Appeals of the Patent Office.

Application for patent of Max J. Kalm, Serial No. 803,847, filed Apr. 3, 1959; Patent Office Group 120. From decision rejecting claims 1 to 3, applicant appeals. Reversed.

HELMUTH A. WEGNER, Chicago, Ill., for appellant.

JOSEPH SCHIMMEL (JACK E. ARMORE of counsel) for Commissioner of Patents.

Before WORLEY, Chief Judge, RICH,

Capon v. Eshhar

**U.S. Court of Appeals
Federal Circuit**

Nos. 03-1480, -1481

Decided August 12, 2005

PATENTS

**[1] Patentability/Validity — Specification
— Written description (§ 115.1103)**

Board of Patent Appeals and Interferences erred in holding that interference parties' specifications for claims directed to chimeric genes designed to enhance immune response do not satisfy written description requirement of 35 U.S.C. § 112, even though specifications do not include complete nucleotide sequences of claimed genes, since written description requirement must be applied in context of particular invention and state of knowledge, and there is no per se rule that nucleotide sequence must be recited anew when that information is already known in art, since invention at issue lies not in discovering which DNA segments are related to immune response, but in novel combination of segments to achieve novel result, since claimed chimeric genes are prepared from known DNA sequences of known function, and since requirement that these sequences be analyzed and reported in specifications therefore does not add descriptive substance.

**[2] Patentability/Validity — Specification
— Written description (§ 115.1103)**

**Patentability/Validity — Specification —
Enablement (§ 115.1105)**

**Patent construction — Claims — Broad
or narrow (§ 125.1303)**

Determination of adequate support for generic claims to biological subject matter depends, among other factors, on existing knowledge in field, extent and content of prior art, maturity of science or technology, predictability of aspect at issue, and other considerations appropriate to claimed subject matter; in present case, Board of Patent Appeals and Interferences erred in failing to address support for each of parties' claims directed to chimeric genes designed to enhance immune response, since parties presented general teachings as to how to select and recombine DNA,

as well as specific examples of production of specified chimeric genes, since they used standard systems of description and identification, as well as known procedures for selecting, isolating, and linking known DNA segments, since board's repeated observation that full scope of claims appears to be "enabled" cannot be reconciled with its objection that only "general plan" to combine unidentified DNA is presented, since whether inventors demonstrated sufficient generality to support scope of claims must be determined claim by claim, and since board's position that inventions at issue were merely "invitation to experiment" did not distinguish among broad and narrow claims, and concerns enablement more than written description.

**Particular patents — Chemical — Chi-
meric genes**

6,407,221, Capon, Weiss, Irving, Roberts, and Zsebo, chimeric chains for receptor-associated signal transduction pathways, cancellation of claims corresponding to count in interference no. 103,887, for failure to satisfy written description requirement, vacated and remanded.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent interference proceeding (no. 103,887) between Daniel J. Capon, Arthur Weiss, Brian A. Irving, Margo R. Roberts, and Krisztina Zsebo (patent no. 6,407,221), and Zelig Eshhar, Daniel Schindler, Tova Waks, and Gideon Gross (application serial no. 08/084,994). Both parties appeal from cancellation of claims corresponding to interference count on ground that neither party met written description requirement. Jon Dudas, in his capacity as Director of the PTO, intervenes in support of the board. Vacated and remanded.

Steven B. Kelber, of Piper Rudnick, Washington, D.C., for appellants.

Roger L. Browdy, of Browdy and Neimark, Washington, for cross-appellants.

Mary L. Kelly, associate solicitor, John M. Whealan, solicitor, and Stephen Walsh, associate solicitor, U.S. Patent and Trademark Office, Arlington, Va., for intervenor.

76 USPQ2d

76 USPQ2d

Capon v. Eshhar

1079

l as specific examples of production of ad chimeric genes, since they used stan- dards of description and identification, l as known procedures for selecting, ig, and linking known DNA segments, board's repeated observation that full of claims appears to be "enabled" can- be reconciled with its objection that only al plan" to combine unidentified DNA- ments, since whether inventors demon- sufficient generality to support scope as must be determined claim by claim, ce board's position that inventions at- ere merely "invitation to experiment" distinguish among broad and narrow and concerns enablement more than descriptio.

Before Newman, Mayer,* and Gajarsa, cir- cuit judges.

Newman, J.

Both of the parties to a patent interference proceeding have appealed the decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Of- fice, wherein the Board held that the specifi- cation of neither party met the written descrip- tion requirement of the patent statute. *Capon v. Eshhar*, Interf. No. 103,887 (Bd. Pat. App. & Interf. Mar. 26, 2003). The Board dissolved the interference and cancelled all of the claims of both parties corresponding to the interfe- rence count. With this ruling, the Board termi- nated the proceeding and did not reach the question of priority of invention. We conclude that the Board erred in its application of the law of written description. The decision is va- cated and the case is remanded to the Board for further proceedings.

BACKGROUND

Daniel J. Capon, Arthur Weiss, Brian A. Irving, Margo R. Roberts, and Krisztina Zsebo (collectively "Capon") and Zelig Eshhar, Daniel Schindler, Tova Waks, and Gideon Gross (collectively "Eshhar") were the par- ties to an interference proceeding between Capon's United States Patent No. 6,407,221 ("the '221 patent") entitled "Chimeric Chains for Receptor-Associated Signal Transduction Pathways" and Eshhar's patent application Serial No. 08/084,994 ("the '994 applica- tion") entitled "Chimeric Receptor Genes and Cells Transformed Therewith." Capon's Patent No. 5,359,046 ("the '046 patent"), par- ent of the '221 patent, was also included in the interference but was held expired for non- payment of a maintenance fee. The PTO in- cluded the '046 patent in its decision and in its argument of this appeal.¹

A patent interference is an administrative proceeding pursuant to 35 U.S.C. §§ 102(g) and 135(a), conducted for the purpose of de- termining which of competing applicants is the first inventor of common subject matter.

* Haldane Robert Meyer vacated the position of Chief Judge on December 24, 2004.

¹ Although Capon is designated as appellant and Eshhar as cross-appellant, both appealed the Board's decision. See Fed. R. App. P. 28(b). The Director of the PTO intervened to support the Board, and has fully par- ticipated in this appeal.

An interference is instituted after the separate patent applications have been examined and found to contain patentable subject matter. Capon's patents had been examined and had issued before this interference was instituted, and Eshhar's application had been examined and allowed but a patent had not yet issued.

During an interference proceeding the Board is authorized to determine not only pri- ority of invention but also to redetermine pa- tentability. 35 U.S.C. § 6(b). The question of patentability of the claims of both parties was raised *sua sponte* by an administrative patent judge during the preliminary proceedings. Thereafter the Board conducted an *inter par- tes* proceeding limited to this question, receiv- ing evidence and argument. The Board then invalidated all of the claims that had been de- signated as corresponding to the count of the interference, *viz.*, all of the claims of the Capon '221 patent, claims 5-8 of the Capon '046 patent, and claims 1-7, 9-20, and 23 of the Eshhar '994 application.

In accordance with the Administrative Pro- cedure Act, the law as interpreted and applied by the agency receives plenary review on ap- peal, and the agency's factual findings are re- viewed to determine whether they were arbi- trary, capricious, or unsupported by substan- tial evidence in the administrative record. See 5 U.S.C. § 706(2); *Dickinson v. Zurko*, 527 U.S. 150, 164-65 [50 USPQ2d 1930] (1999); *In re Gartside*, 203 F.3d 1305, 1315 (53 USPQ2d 1769) (Fed. Cir. 2000).

The Invention

A chimeric gene is an artificial gene that combines segments of DNA in a way that does not occur in nature. The '221 patent and '994 application are directed to the production of chimeric genes designed to enhance the im- mune response by providing cells with spec- ific cell-surface antibodies in a form that can penetrate diseased sites, such as solid tumors, that were not previously reachable. The par- ties explain that their invention is a way of en- dowing immune cells with antibody-type specificity, by combining known antigen- binding-domain producing DNA and known lymphocyte-receptor-protein producing DNA into a unitary gene that can express a unitary polypeptide chain. Eshhar summarized the problem to which the invention is directed:

Antigen-specific effector lymphocytes, such as tumor-specific T cells, are very rare.

cultural genes — Chemical — Chi- ric genes

.221, Capon, Weiss, Irving, Roberts, cho, chimeric chains for receptor- ed signal transduction pathways, can- of claims corresponding to count in nce no. 103,887, for failure to satisfy description requirement, vacated and d.

l from the U.S. Patent and Trademark board of Patent Appeals and Interfe-

interference proceeding (no. between Daniel J. Capon, Arthur rian A. Irving, Margo R. Roberts, and i Zsebo (patent no. 6,407,221), and hhar, Daniel Schindler, Tova Waks, on Gross (application serial no. 08/ Both parties appeal from cancella- claims corresponding to interference ground that neither party met written n requirement. Jon Dudas, in his ca- Director of the PTO, intervenes in f the board. Vacated and remanded.

B. Kelber, of Piper Rudnick, Wash- .C., for appellants.

L. Browdy, of Browdy and Neimark, on, for cross-appellants.

.. Kelly, associate solicitor, John M. solicitor, and Stephen Walsh, asso- sitor, U.S. Patent and Trademark Of- ington, Va., for intervenor.

individual-specific, limited in their recognition spectrum and difficult to obtain against most malignancies. Antibodies, on the other hand, are readily obtainable, more easily derived, have wider spectrum and are not individual-specific. The major problem of applying specific antibodies for cancer immunotherapy lies in the inability of sufficient amounts of monoclonal antibodies (mAb) to reach large areas within solid tumors.

Technical Paper Explaining Eshhar's Invention, at 6.

The inventions of Capon and Eshhar are the chimeric DNA that encodes single-chain chimeric proteins for expression on the surface of cells of the immune system, plus expression vectors and cells transformed by the chimeric DNA. The experts for both parties explain that the invention combines selected DNA segments that are both endogenous and nonendogenous to a cell of the immune system, whereby the nonendogenous segment encodes the single-chain variable ("scFv") domain of an antibody, and the endogenous segment encodes cytoplasmic, transmembrane, and extracellular domains of a lymphocyte signaling protein. They explain that the scFv domain combines the heavy and light variable ("Fv") domains of a natural antibody, and thus has the same specificity as a natural antibody. Linking this single chain domain to a lymphocyte signaling protein creates a chimeric scFv-receptor ("scFvR") gene which, upon transfection into a cell of the immune system, combines the specificity of an antibody with the tissue penetration, cytokine production, and target-cell destruction capability of a lymphocyte.

The parties point to the therapeutic potential if tumors can be infiltrated with specifically designed immune cells of appropriate anti-tumor specificity.

The Eshhar Claims

The Board held unpatentable the following claims of Eshhar's '994 application; these were all of the '994 claims that had been designated as corresponding to the count of the interference. Eshhar's claim 1 was the designated count.

1. A chimeric gene comprising

a first gene segment encoding a single-chain Fv domain (scFv) of a specific antibody and

a second gene segment encoding partially or entirely the transmembrane and cytoplasmic, and optionally the extracellular, domains of an endogenous protein

wherein said endogenous protein is expressed on the surface of cells of the immune system and triggers activation and/or proliferation of said cells.

which chimeric gene, upon transfection to said cells of the immune system, expresses said scFv domain and said domains of said endogenous protein in one single chain on the surface of the transfected cells such that the transfected cells are triggered to activate and/or proliferate and have MHC nonrestricted antibody-type specificity when said expressed scFv domain binds to its antigen.

2. A chimeric gene according to claim 1 wherein the second gene segment further comprises partially or entirely the extracellular domain of said endogenous protein.

3. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against tumor cells.

4. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against virus infected cells.

5. A chimeric gene according to claim 4 wherein the virus is HIV.

6. A chimeric gene according to claim 1 wherein the second gene segment encodes a lymphocyte receptor chain.

7. A chimeric gene according to claim 6 wherein the second gene segment encodes a chain of the T cell receptor.

9. A chimeric gene according to claim 7 wherein the second gene segment encodes the α , β , γ , or δ chain of the antigen-specific T cell receptor.

10. A chimeric gene according to claim 1 wherein the second gene segment encodes a polypeptide of the TCR/CD3 complex.

11. A chimeric gene according to claim 10 wherein the second gene segment encodes the zeta or eta isoform chain.

12. A chimeric gene according to claim 1 wherein the second gene segment encodes a subunit of the Fc receptor or IL-2 receptor.

13. A
wherein
comm
recept
14. A
wherein
15. A
where
the C
16. A
where
the α
17. A
meric
18. A
with
an ex
19. A
with
meric
20. A
to clai
ing of
tivates
helped
23. A
where
phocy
TCR/
or IL-
The F
rately, a
satisfy
to all of
The Ca
Clair
patent,
scriptio
the chin
corres-
claim 9.
1. A
houn
ing it
whic
to the
extra

second gene segment encoding part entirely the transmembrane and cy-
mic, and optionally the extracellular,
ns of an endogenous protein

herein said endogenous protein is ex-
d on the surface of cells of the im-
system and triggers activation and/or
ration of said cells,

which chimeric gene, upon transfec-
said cells of the immune system, ex-
said scFv domain and said domains
d endogenous protein in one single
on the surface of the transfected cells
hat the transfected cells are triggered
tivate and/or proliferate and have
nonrestricted antibody-type specifi-
cien said expressed scFv domain binds
antigen.

chimeric gene according to claim 1
in the second gene segment further
xes partially or entirely the extracel-
lomain of said endogenous protein.

chimeric gene according to claim 1
in the first gene segment encodes the
domain of an antibody against tumor

chimeric gene according to claim 1
in the first gene segment encodes the
domain of an antibody against virus
ad cells.

chimeric gene according to claim 4
in the virus is HIV.

chimeric gene according to claim 1
in the second gene segment encodes a
ocyte receptor chain.

chimeric gene according to claim 6
in the second gene segment encodes a
of the T cell receptor.

chimeric gene according to claim 7
in the second gene segment encodes
, β , γ , or δ chain of the antigen-
ic T cell receptor.

chimeric gene according to claim 1
in the second gene segment encodes a
epitope of the TCR/CD3 complex.

chimeric gene according to claim 10
in the second gene segment encodes
ta or eta isoform chain.

chimeric gene according to claim 1
in the second gene segment encodes a
it of the Fc receptor or IL-2 receptor.

13. A chimeric gene according to claim 12
wherein the second gene segment encodes a
common subunit of IgE and IgG binding Fc
receptors;

14. A chimeric gene according to claim 13
wherein said subunit is the gamma subunit.

15. A chimeric gene according to claim 13
wherein the second gene segment encodes
the CD16a chain of the Fc γ RIII or Fc γ RII.

16. A chimeric gene according to claim 12
wherein the second gene segment encodes
the α or β subunit of the IL-2 receptor.

17. An expression vector comprising a chi-
meric gene according to claim 1.

18. A cell of the immune system endowed
with antibody specificity transformed with
an expression vector according to claim 17.

19. A cell of the immune system endowed
with antibody specificity comprising a chi-
meric gene according to claim 1.

20. A cell if the immune system according
to claim 19 selected from the group consist-
ing of a natural killer cell, a lymphokine ac-
tivated killer cell, a cytotoxic T cell, a
helper T cell and a subtype thereof.

23. A chimeric gene according to claim 1
wherein said endogenous protein is a lym-
phocyte receptor chain, a polypeptide of the
TCR/CD3 complex, or a subunit of the Fc
or IL-2 receptor.

The Board did not discuss the claims sepa-
rately, and held that the specification failed to
satisfy the written description requirement as
to all of these claims.

The Capon Claims

Claims 1-10, all of the claims of the '221
patent, were held unpatentable on written de-
scription grounds. Claims 1-6 are directed to
the chimeric DNA, claims 7, 8, and 10 to the
corresponding cell comprising the DNA, and
claim 9 to the chimeric protein:

1. A chimeric DNA encoding a membrane
bound protein, said chimeric DNA compris-
ing in reading frame:

DNA encoding a signal sequence
which directs said membrane bound protein
to the surface membrane;

DNA encoding a non-MHC restricted
extracellular binding domain which is ob-

tained from a single chain antibody that
binds specifically to at least one ligand,
wherein said at least one ligand is a protein
on the surface of a cell or a viral protein;

DNA encoding a transmembrane do-
main which is obtained from a protein se-
lected from the group consisting of CD4,
CD8, immunoglobulin, the CD3 zeta chain,
the CD3 gamma chain, the CD3 delta chain
and the CD3 epsilon chain; and

DNA encoding a cytoplasmic signal-
transducing domain of a protein that acti-
vates an intracellular messenger system
which is obtained from CD3 zeta.

wherein said extracellular domain and
said cytoplasmic domain are not naturally
joined together, and said cytoplasmic do-
main is not naturally joined to an extracel-
lular ligand-binding domain, and when said
chimeric DNA is expressed as a membrane
bound protein in a host cell under condi-
tions suitable for expression, said mem-
brane bound protein initiates signaling in
said host cell when said extracellular do-
main binds said at least one ligand.

2. The DNA of claim 1, wherein said
single-chain antibody recognizes an antigen
selected from the group consisting of viral
antigens and tumor cell associated antigens.

3. The DNA of claim 2 wherein said single-
chain antibody is specific for the HIV env
glycoprotein.

4. The DNA of claim 1, wherein said trans-
membrane domain is naturally joined to
said cytoplasmic domain.

5. An expression cassette comprising a tran-
scriptional initiation region, the DNA of
claim 1 under the transcriptional control of
said transcriptional initiation region, and a
transcriptional termination region.

6. A retroviral RNA or DNA construct com-
prising the expression cassette of claim 5.

7. A cell comprising the DNA of claim 1.

8. The cell of claim 7, wherein said cell is
a human cell.

9. A chimeric protein comprising in the
N-terminal to C-terminal direction:

a non-MHC restricted extracellular
binding domain which is obtained from a
single chain antibody that binds specifically
to at least one ligand, wherein said at least

one ligand is a protein on the surface of a cell or a viral protein;

a transmembrane domain which is obtained from a protein selected from the group consisting of CD4, CD8, immunoglobulin, the CD3 zeta chain, the CD3 gamma chain, the CD3 delta chain and the CD3 epsilon chain; and

a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system which is obtained from CD3 zeta,

wherein said extracellular domain and said cytoplasmic domain are not naturally joined together, and said cytoplasmic domain is not naturally joined to an extracellular ligand-binding domain, and when said chimeric protein is expressed as a membrane bound protein in a host cell under conditions suitable for expression, said membrane bound protein initiates signaling in said host cell when said extracellular domain binds said at least one ligand.

10. A mammalian cell comprising as a surface membrane protein, the protein of claim 9.

In addition, claims 5, 6, 7, and 8 of Capon's '046 patent were held unpatentable. These claims are directed to chimeric DNA sequences where the encoded extracellular domain is a single-chain antibody containing ligand binding activity.

The Board Decision

The Board presumed enablement by the specifications of the '221 patent and '994 application of the full scope of their claims, and based its decision solely on the ground of failure of written description. The Board held that neither party's specification provides the requisite description of the full scope of the 'chimeric DNA or encoded proteins, by reference to knowledge in the art of the "structure, formula, chemical name, or physical properties" of the DNA or the proteins. In the Board's words:

We are led by controlling precedent to understand that the full scope of novel chimeric DNA the parties claim is not described in their specifications under 35 U.S.C. § 112, first paragraph, by reference to contemporary and/or prior knowledge in the art of the structure, formula, chemical

name, or physical properties of many protein domains, and/or DNA sequences which encode many protein domains, which comprise single-chain proteins and/or DNA constructs made in accordance with the plans, schemes, and examples thereof the parties disclose.

Bd. op. at 4. As controlling precedent the Board cited *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559 [43 USPQ2d 1398] (Fed. Cir. 1997); *Fiers v. Revel Co.*, 984 F.2d 1164 [25 USPQ2d 1601] (Fed. Cir. 1993); *Angen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200 [18 USPQ2d 1016] (Fed. Cir. 1991); and *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316 [63 USPQ2d 1609] (Fed. Cir. 2002). The Board summarized its holding as follows:

Here, both Eshhar and Capon claim novel genetic material described in terms of the functional characteristics of the protein it encodes. Their specifications do not satisfy the written description requirement because persons having ordinary skill in the art would not have been able to visualize and recognize the identity of the claimed genetic material without considering additional knowledge in the art, performing additional experimentation, and testing to confirm results.

Bd. op. at 89.

DISCUSSION

Eshhar and Capon challenge both the Board's interpretation of precedent and the Board's ruling that their descriptions are inadequate. Both parties explain that their chimeric genes are produced by selecting and combining known heavy- and light-chain immune-related DNA segments, using known DNA-linking procedures. The specifications of both parties describe procedures for identifying and obtaining the desired immune-related DNA segments and linking them into the desired chimeric genes. Both parties point to their specific examples of chimeric DNA prepared using identified known procedures, along with citation to the scientific literature as to every step of the preparative method.

The parties presented expert witnesses who placed the invention in the context of prior knowledge and explained how the descriptive text would be understood by persons of skill in the field of the invention. The witnesses ex-

name, or physical properties of many protein domains, and/or DNA sequences which encode many protein domains, which comprise single-chain proteins and/or DNA constructs made in accordance with the plans, schemes, and examples thereof the parties disclose.

Bd. op. at 4. As controlling precedent the Board cited *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559 [43 USPQ2d 1398] (Fed. Cir. 1997); *Fiers v. Revel Co.*, 984 F.2d 1164 [25 USPQ2d 1601] (Fed. Cir. 1993); *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200 [18 USPQ2d 1016] (Fed. Cir. 1991); and *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316 [63 USPQ2d 1609] (Fed. Cir. 2002). The Board summarized its holding as follows:

Here, both Eshhar and Capon claim novel genetic material described in terms of the functional characteristics of the protein it encodes. Their specifications do not satisfy the written description requirement because persons having ordinary skill in the art would not have been able to visualize and recognize the identity of the claimed genetic material without considering additional knowledge in the art, performing additional experimentation, and testing to confirm results.

Bd. op. at 89.

DISCUSSION

Eshhar and Capon challenge both the Board's interpretation of precedent and the Board's ruling that their descriptions are inadequate. Both parties explain that their chimeric genes are produced by selecting and combining known heavy- and light-chain immune-related DNA segments, using known DNA-linking procedures. The specifications of both parties describe procedures for identifying and obtaining the desired immune-related DNA segments and linking them into the desired chimeric genes. Both parties point to their specific examples of chimeric DNA prepared using identified known procedures, along with citation to the scientific literature as to every step of the preparative method.

The parties presented expert witnesses who placed the invention in the context of prior knowledge and explained how the descriptive text would be understood by persons of skill in the field of the invention. The witnesses ex-

plained that the principle of forming chimeric genes from selected segments of DNA was known, as well as their methods of identifying, selecting, and combining the desired segments of DNA. Dr. Eshhar presented an expert statement wherein he explained that the prior art contains extensive knowledge of the nucleotide structure of the various immune-related segments of DNA; he stated that over 785 mouse antibody DNA light chains and 1,327 mouse antibody DNA heavy chains were known and published as early as 1991. Similarly Capon's expert Dr. Desiderio discussed the prior art, also citing scientific literature:

The linker sequences disclosed in the '221 patent (col. 24, lines 4 and 43) used to artificially join a heavy and light chain nucleic acid sequence and permit functional association of the two ligand binding regions were published by 1990, as were the methods for obtaining the mature sequences of the desired heavy and light chains for constructing a SAh (Exhibit 47, Batra et al., J. Biol. Chem., 1990; Exhibit 48, Bird et al., Science, 1988; Exhibit 50, Huston et al., PNAS, 1988; Exhibit 51, Chaudhary, PNAS, 1990; Exhibit 56, Morrison et al., Science, 1985; Exhibit 53, Sharon et al., Nature 1984).

Desiderio declaration at 4 ¶ 11.

Both parties stated that persons experienced in this field would readily know the structure of a chimeric gene made of a first segment of DNA encoding the single-chain variable region of an antibody, and a second segment of DNA encoding an endogenous protein. They testified that re-analysis to confirm these structures would not be needed in order to know the DNA structure of the chimeric gene, and that the Board's requirement that the specification must reproduce the "structure, formula, chemical name, or physical properties" of these DNA combinations had been overtaken by the state of the science. They stated that where the structure and properties of the DNA components were known, re-analysis was not required.

Eshhar's specification contains the nucleotide sequences of sixteen different receptor primers and four different scFv primers from which chimeric genes encoding scFvR may be obtained, while Capon's specification cites literature sources of such information. Eshhar's specification shows the production of chi-

meric genes encoding scFvR using primers, as listed in Eshhar's Table 1. Capon stated that natural genes are isolated and joined using conventional methods, such as the polymerase chain reaction or cloning by primer repair. Capon, like Eshhar, discussed various known procedures for identifying, obtaining, and linking DNA segments, accompanied by experimental examples. The Board did not dispute that persons in this field of science could determine the structure or formula of the linked DNA from the known structure or formula of the components.

The Board stated that "controlling precedent" required inclusion in the specification of the complete nucleotide sequence of "at least one" chimeric gene. Bd. op. at 4. The Board also objected that the claims were broader than the specific examples. Eshhar and Capon each responds by pointing to the scientific completeness and depth of their descriptive texts, as well as to their illustrative examples. The Board did not relate any of the claims, broad or narrow, to the examples, but invalidated all of the claims without analysis of their scope and the relation of claim scope to the details of the specifications.

Eshhar and Capon both argue that they have set forth an invention whose scope is fully and fairly described, for the nucleotide sequences of the DNA in chimeric combination is readily understood to contain the nucleotide sequences of the DNA components. Eshhar points to the general and specific description in his specification of known immune-related DNA segments, including the examples of their linking. Capon points similarly to his description of selecting DNA segments that are known to express immune-related proteins, and stresses the existing knowledge of these segments and their nucleotide sequences, as well as the known procedures for selecting and combining DNA segments, as cited in the specification.

Both parties argue that the Board misconstrued precedent, and that precedent does not establish a *per se* rule requiring nucleotide-by-nucleotide re-analysis when the structure of the component DNA segments is already known, or readily determined by known procedures.

The Statutory Requirement

The required content of the patent specification is set forth in Section 112 of Title 35:

§ 112 ¶ 1. The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. See *Enzo Biochem*, 296 F.3d at 1330 (the written description requirement "is the quid pro quo of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reljin v. Microsoft Corp.*, 214 F.3d 1342, 1345-46 [54 USPQ2d 1915] (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker*, 559 F.2d 588, 592 n.4 [194 USPQ 470] (CCPA 1977) (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

For the chimeric genes of the Capon and Eshhar inventions, the law must take cognizance of the scientific facts. The Board erred in refusing to consider the state of the scientific knowledge, as explained by both parties, and in declining to consider the separate scope of each of the claims. None of the cases to which the Board attributes the requirement of total DNA re-analysis, i.e., *Regents v. Lilly*, *Fiers v. Revel*, *Amgen*, or *Enzo Biochem*, require a re-description of what was already known. In *Lilly*, 119 F.3d at 1567, the cDNA for human insulin had never been characterized. Similarly in *Fiers*, 984 F.2d at 1171, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In *Amgen*, 927 F.2d at 1206, the court explained that a novel gene was not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In *Enzo Biochem*, 296 F.3d at 1326, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332 [65 USPQ2d 1385] (Fed. Cir. 2003) the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." These evolving principles were applied in *Noelle v. Lederman*, 355 F.3d 1343, 1349 [69 USPQ2d 1508] (Fed. Cir. 2004), where the court affirmed that the human antibody there at issue was not adequately described by the structure and function of the mouse antigen; and in *University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 925-26 [69 USPQ2d 1886] (Fed. Cir. 2004), where the court affirmed that the description of the COX-2 enzyme did not serve to describe unknown compounds capable of selectively inhibiting the enzyme.

[1] The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set

a per se terminated person expert would know would be linked by plain that which D8 mune resp. in the no ments to 2

The states that vention; it must be d field evol (twen whi each inven Capon ex concern th structure, d at issue at quences of quirement lyzed and not add c erred in ho meet the w cause they mula or ch quences of

Claim Sco

There rel specification. of all of the rector argue all of the pe ered by the tenced purg broad becau species. The vided an ad cation of th stood by pe They state t vary, and th evaluation (c meric combi It is well, able" fields, ognize the y rning the s inventor is t focuses on t cation. See,

76 USPQ2d

76 USPQ2d

Capon v. Eshhar

1085

chimeric genes of the Capon and ventions, the law must take cognitive scientific facts. The Board erred to consider the state of the science, as explained by both parties, defining to consider the separate scope of the claims. None of the cases to Board attributes the requirement of a re-analysis, i.e., *Regents v. Lilly*, level, *Amgen*, or *Enzo Biochem*, re-description of what was already in *Lilly*, 119 F.3d at 1567, the cDNA a insulin had never been characterized in *Fiers*, 984 F.2d at 1171, the DNA sought to be claimed was own structure, whereby this court breadth of the claims as embracing "or research "plan." In *Amgen*, at 1206, the court explained that a re was not adequately characterized logical function alone because such ion would represent a mere "wish to identity" of the novel material. In *them*, 296 F.3d at 1326, this court rehad deposit of a physical sample may vords when description is beyond scientific capability. In *Amgen Inc. v. dation Roussel, Inc.*, 314 F.3d 1313, USPQ2d 13851 (Fed. Cir. 2003) the claimed further that the written requirement may be satisfied "if in ledge of the art the disclosed functionally correlated to a particular, ructure." These evolving principles aid in *Noelle v. Lederman*, 355 F.3d 49 [69 USPQ2d 1508] (Fed. Cir. here the court affirmed that the hubody there at issue was not ad-described by the structure and function mouse antigen; and in *University of v. G.D. Searle & Co.*, 358 F.3d 916, 59 USPQ2d 18861 (Fed. Cir. 2004), court affirmed that the description CX-2 enzyme did not serve to de-known compounds capable of selecting the enzyme.

3 "written description" requirement applied in the context of the particular and the state of the knowledge, d's rule that the nucleotide sequences meric genes must be fully presented, the nucleotide sequences of the cDNA are known, is an inappropriate action. When the prior art includes the e information, precedent does not set

a *per se* rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.

The "written description" requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution. Both Eshhar and Capon explain that this invention does not concern the discovery of gene function or structure, as in *Lilly*. The chimeric genes here at issue are prepared from known DNA sequences of known function. The Board's requirement that these sequences must be analyzed and reported in the specification does not add descriptive substance. The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes.

Claim Scope

There remains the question of whether the specifications adequately support the breadth of all of the claims that are presented. The Director argues that it cannot be known whether all of the permutations and combinations covered by the claims will be effective for the intended purpose, and that the claims are too broad because they may include inoperative species. The inventors say that they have provided an adequate description and exemplification of their invention as would be understood by persons in the field of the invention. They state that biological properties typically vary, and that their specifications provide for evaluation of the effectiveness of their chimeric combinations.

It is well recognized that in the "unpredictable" fields of science, it is appropriate to recognize the variability in the science in determining the scope of the coverage to which the inventor is entitled. Such a decision usually focuses on the exemplification in the specification. See, e.g., *Enzo Biochem*, 296 F.3d at

1327-28 (remanding for district court to determine "[w]hether the disclosure provided by the three deposits in this case, coupled with the skill of the art, describes the genera of claims 1-3 and 5"); *Lilly*, 119 F.3d at 1569 (genus not described where "a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus" had not been provided); *In re Gostelli*, 872 F.2d 1008, 1012 [10 USPQ2d 1614] (Fed. Cir. 1989) (two chemical compounds were insufficient description of subgenus); *In re Smith*, 458 F.2d 1389, 1394-95 [173 USPQ 679] (CCPA 1972) (disclosure of genus and one species was not sufficient description of intermediate subgenus); *In re Grimmer*, 274 F.2d 949, 952 [124 USPQ 499] (CCPA 1960) (disclosure of single example and statement of scope sufficient disclosure of subgenus).

[2] Precedent illustrates that the determination of what is needed to support generic claims to biological subject matter depends on a variety of factors, such as the existing knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter. See, e.g., *In re Wallach*, 378 F.3d 1330, 1333-34 [71 USPQ2d 1939] (Fed. Cir. 2004) (an amino acid sequence supports "the entire genus of DNA sequences" that can encode the amino acid sequence because "the state of the art has developed" such that it is a routine matter to convert one to the other); *University of Rochester*, 358 F.3d at 925 (considering whether the patent disclosed the compounds necessary to practice the claimed method, given the state of technology); *Singh v. Bruce*, 317 F.3d 1334, 1343 [65 USPQ2d 1641] (Fed. Cir. 2002) (affirming adequacy of disclosure by distinguishing precedent in which the selection of a particular species within the claimed genus had involved "highly unpredictable results").

It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. See *In re Angstadt*, 537 F.2d 498, 504 [190 USPQ 214] (CCPA 1976) ("The examples, both operative and inoperative, are the best guidance this art permits, as far as we can conclude from the record"). While the Board is correct that a generic invention re-

quires adequate support, the sufficiency of the support must be determined in the particular case. Both Eshhar and Capon present not only general teachings of how to select and recombine the DNA, but also specific examples of the production of specified chimeric genes. For example, Eshhar points out that in Example 1 of his specification the FcR γ chain was used, which chain was amplified from a human cDNA clone, using the procedure of Kuster, H. et al., *J. Biol. Chem.*, 265:6448-6451 (1990), which is cited in the specification and reports the complete sequence of the FcR γ chain. Eshhar's Example 1 also explains the source of the genes that provide the heavy and light chains of the single chain antibody, citing the PhD thesis of Gideon Gross, a co-inventor, which cites a reference providing the complete sequence of the Sp6 light chain gene used to construct the single-chain antibody. Eshhar states that the structure of the Sp6 heavy chain antibody was well known to those of skill in the art and readily accessible on the internet in a database as entry EM-BL:MMSP6718, Example 5 at page 54 of the Eshhar specification cites Ravetch et al., *J. Exp. Med.*, 170:481-497 (1989) for the method of producing the CD16 α DNA clone that was PCR amplified; this reference published the complete DNA sequence of the CD16 α chain, as discussed in paragraph 43 of the Eshhar Declaration. Example 3 of the Eshhar specification uses the DNA of the monoclonal anti-HER2 antibody and states that the N29 hybridoma that produces this antibody was deposited with the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, on August 19, 1992, under Deposit No. CNCM I-1262. It is incorrect to criticize the methods, examples, and referenced prior art of the Eshhar specification as but "a few PCR primers and probes," as does the Director's brief.

Capon's Example 3 provides a detailed description of the creation and expression of single chain antibody fused with T-cell receptor zeta chain, referring to published vectors and procedures. Capon, like Eshhar, describes gene segments and their ligation to form chimeric genes. Although Capon includes fewer specific examples in his specification than does Eshhar, both parties used standard systems of description and identification, as well as known procedures for selecting, isolating, and linking known DNA segments. Indeed,

the Board's repeated observation that the full scope of all of the claims appears to be "enabled" cannot be reconciled with the Board's objection that only a "general plan" to combine unidentified DNA is presented. *See In re Wands*, 858 F.2d 731, 736-37 [8 USPQ2d 1400] (Fed. Cir. 1988) (experimentation to practice invention must not be "undue" for invention to be considered enabled).

The PTO points out that for biochemical processes relating to gene modification, protein expression, and immune response, success is not assured. However, generic inventions are not thereby invalid. Precedent distinguishes among generic inventions that are adequately supported, those that are merely a "wish" or "plan," the words of *Fiers v. Revel*, 984 F.2d at 1171, and those in between, as illustrated by *Noelle v. Lederman*, 355 F.3d at 1350; the facts of the specific case must be evaluated. The Board did not discuss the generic concept that both Capon and Eshhar described — the concept of selecting and combining a gene sequence encoding the variable domain of an antibody and a sequence encoding a lymphocyte activation protein, into a single DNA sequence which, upon expression, allows for immune responses that do not occur in nature. The record does not show this concept to be in the prior art, and includes experimental verification as well as potential variability in the concept.

Whether the inventors demonstrated sufficient generality to support the scope of some or all of their claims, must be determined claim by claim. The Board did not discuss the evidence with respect to the generality of the invention and the significance of the specific examples, instead simply rejecting all the claims for lack of a complete chimeric DNA sequence. As we have discussed, that reasoning is inapt for this case. The Board's position that the patents at issue were merely an "invitation to experiment" did not distinguish among the parties' broad and narrow claims, and further concerns enablement more than written description. *See Adang v. Fischhoff*, 286 F.3d 1346, 1355 [62 USPQ2d 1504] (Fed. Cir. 2002) (enablement involves assessment of whether one of skill in the art could make and use the invention without undue experimentation); *In re Wright*, 999 F.2d 1557, 1561 [27 USPQ2d 1510] (Fed. Cir. 1993) (same). Although the legal criteria of enablement and written description are related and are often

d's repeated observation that the full
all of the claims appears to be "en-
cannot be reconciled with the Board's
n that only a "general plan" to re-
comfidential DNA is presented. See *In re*
858 F.2d 731, 736-37 [8 USPQ2d
Fed. Cir. 1988] (experimentation to
invention must not be "undue" for
n to be considered enabled).

TO points out that for biochemical
s relating to gene modification, pro-
gression, and immune response, suc-
not assured. However, generic inven-
not thereby invalid. Precedent distin-
among generic inventions that are ad-
supported, those that are merely a
or "plan," the words of *Fiers v. Revel*,
1 at 1171, and those in between, as il-
l by *Noelle v. Lederman*, 355 F.3d at
in facts of the specific case must be
d. The Board did not discuss the ge-
concept that both Capon and Eshbar de-
— the concept of selecting and com-
gene sequence encoding the variable
of an antibody and a sequence encod-
mpocyte activation protein, into a
NA sequence which, upon expression,
or immune responses that do not oc-
cure. The record does not show this
to be in the prior art, and includes ex-
tal verification as well as potential
ty in the concept.

her the inventors demonstrated suffi-
nerality to support the scope of some
of their claims, must be determined
claim. The Board did not discuss the
e with respect to the generality of the
n and the significance of the specific
s, instead simply rejecting all the
or lack of a complete chimeric DNA
e. As we have discussed, that reason-
apt for this case. The Board's position
patents at issue were merely an "invi-
o experiment" did not distinguish
the parties' broad and narrow claims,
ther concerns enablement more than
description. See *Adang v. Fischhoff*,
1346, 1355 [62 USPQ2d 1504] (Fed.
2) (enablement involves assessment of
one of skill in the art could make and
invention without undue experimenta-
i re *Wright*, 999 F.2d 1557, 1561 [27
1510] (Fed. Cir. 1993) (same). Al-
the legal criteria of enablement and
description are related and are often

met by the same disclosure, they serve dis-
crete legal requirements.

The predictability or unpredictability of the
science is relevant to deciding how much ex-
perimental support is required to adequately
describe the scope of an invention. Our prede-
cessor court summarized in *In re Storr*, 245
F.2d 474, 478 [114 USPQ 293] (CCPA 1957)
that "[i]t must be borne in mind that, while it
is necessary that an applicant for a patent give
to the public a complete and adequate disclo-
sure in return for the patent grant, the cer-
tainly required of the disclosure is not greater
than that which is reasonable, having due re-
gard to the subject matter involved." This as-
pect may warrant exploration on remand.

In summary, the Board erred in ruling that
§ 112 imposes a *per se* rule requiring recita-
tion in the specification of the nucleotide se-
quence of claimed DNA, when that sequence
is already known in the field. However, the
Board did not explore the support for each of
the claims of both parties, in view of the spe-
cific examples and general teachings in the
specifications and the known science, with ap-
plication of precedent guiding review of the
scope of claims.

We remand for appropriate further proceed-
ings.

VACATED AND REMANDED

NuCar Consulting Inc. v. Doyle

Delaware Chancery Court

No. 19756-NC

Decided April 5, 2005

TRADEMARKS AND UNFAIR TRADE PRACTICES

[1] Trade secrets — Elements of trade se-
cret (§ 400.03)

Trade secrets — State and common law
(§ 400.09)

Under Delaware law, technique or process
will not qualify as trade secret if it is gen-
erally known or readily ascertainable by proper
means; in present case, plaintiff's "Rewards
Program," through which customers of auto-
mobile dealerships earn points toward new
cars or gift certificates with each use of key

tag or other token, is not trade secret within
meaning of Delaware Trade Secrets Act, 6
Del. Code § 2001(4), since component of pro-
gram that allows customer to earn points, and
component involving partnership marketing
with local merchants, are published in bro-
chures and handbooks given to customers who
purchase cars at relevant dealerships, and
plaintiff has made no effort to maintain se-
crecy of alleged trade secrets outlined in
handbooks and brochures, and since plaintiff
contends that manner of communicating data
between plaintiff and dealerships constitutes
trade secret, but does not allege that defend-
ants misappropriated plaintiff's software or
database management system.

[2] Trade secrets — Elements of trade se-
cret (§ 400.03)

Trade secrets — State and common law
(§ 400.09)

Plaintiff's list of potential clients for its cus-
tomer loyalty program, which is internal
document not in public domain, derives inde-
pendent economic value from its secrecy, and
therefore qualifies as trade secret, since por-
tion of list consisting of automotive or motor-
cycle dealerships was developed through in-
terest expressed to plaintiff during national au-
tomobile dealers' convention exhibit, and
through responses from annual mailers, since
plaintiff expended \$45,000 to \$80,000 each
year to develop dealer list, and since, without
similar expenditure of time and money, defend-
ant engaging in same business would have
been unable to discern which dealerships had
interest in purchasing customer loyalty pro-
gram; plaintiff's failure to designate potential
client list as confidential during discovery
does not significantly undermine plaintiff's
trade secret claim, since plaintiff seeks dam-
ages rather than injunctive relief, and there is
no evidence that list was disclosed to or used
by anyone other than defendant during rel-
evant time period.

[3] Trade secrets — Elements of trade se-
cret (§ 400.03)

Trade secrets — Disclosure and misap-
propriation (§ 400.07)

Defendant's use of plaintiff's form contract,
which plaintiff uses to provide dealers with its
customer loyalty program, constitutes misap-

United States Court of Appeals for the Federal Circuit

2008-1079
(Serial No. 08/469,749)

IN RE KENNETH ALONSO

Jennifer A. Johnson, Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., of Washington, DC, argued for appellant. With her on the brief was Susan H. Griffen.

Janet A. Gongola, Associate Solicitor, Office of the Solicitor, United States Patent and Trademark Office, of Arlington, Virginia, argued for the Director of the United States Patent and Trademark Office. With her on the brief was Thomas W. Krause, Associate Solicitor. Of counsel was Sydney O. Johnson, Jr., Acting Solicitor.

Appealed from: United States Patent and Trademark Office
Board of Patent Appeals and Interferences

United States Court of Appeals for the Federal Circuit

2008-1079
(Serial No. 08/469,749)

IN RE KENNETH ALONSO

Appeal from the United States Patent and Trademark Office, Board of Patent Appeals and Interferences in Appeal No. 2006-2148.

DECIDED: October 30, 2008

Before MICHEL, Chief Judge, MAYER, Circuit Judge, and STEARNS,* District Judge.
STEARNS, District Judge.

Dr. Kenneth Alonso appeals a decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences ("Board") sustaining in part the examiner's final rejection of claim 92 of U.S. Patent Application No. 08/469,749 ("749 Application"). In its decision, the Board reversed the examiner's rejection of claim 92 for lack of enablement and sustained the rejection as invalid for lack of adequate written description. Ex parte Alonso, No. 2006-2148 (B.P.A.I. July 25, 2007) ("Decision"). We affirm.

I. BACKGROUND

* Honorable Richard G. Stearns, District Judge, United States District Court for the District of Massachusetts, sitting by designation.

An arsenal of antibodies generated by the immune system defends the human body against illnesses caused by bacteria and cancerous cells and other invasive agents. Antibodies are large, Y-shaped molecules secreted by white blood cells known as "B lymphocytes," or "B-cells." Antibodies are capable of binding to the surfaces of foreign cells or other substances known as "antigens." The specific location on the surface of the antigen where the antibody attaches is termed the "epitope." The arms of the Y-shaped molecule bind to the epitope with specificity. Antibodies that bind to the same epitope are said to have the same "idiotypic." Monoclonal antibodies ("MAbs") are derived from a single precursor and have a single idiotypic. They are produced using "hybridoma" (fusion) technology. A human-to-human hybridoma is created by fusing a human tumor cell to an antibody-producing human B-cell, resulting in secretion by the B-cell of monoclonal antibodies with identical affinity and specificity to a given epitope on the surface of the tumor cell.

On June 6, 1995, Dr. Alonso filed the '749 Application entitled, "Method of Producing Human-Human Hybridomas, The Production of Monoclonal and Polyclonal Antibodies Therefrom, and Therapeutic Use Thereof."¹ The claimed invention recites a method for treating neurofibrosarcoma, a rare cancer of the sheath of a peripheral nerve, that uses human monoclonal antibodies targeted at a patient's tumor. Claim 92 of the '749 Application discloses

[a] method of treating neurofibrosarcoma in a human by administering an effective amount of a monoclonal antibody idiotypic to the neurofibrosarcoma of said human, wherein said monoclonal antibody is secreted from a human-human hybridoma derived from the neurofibrosarcoma cells.

¹ Alonso claimed priority to an application he filed seven years earlier involving similar subject matter.

In Example 1 of the '749 Specification, Alonso described the preparation of a tumor cell suspension from the sample of a tumor and the subsequent sensitization of human spleen cells. The sensitized spleen cells are fused with an immortalized cell line (e.g., a fetal marrow line, a lymphoblastoid line, or a plasma cell line from myeloma). The resulting cells are screened for hybridomas that secrete antibodies specifically reactive with the sensitizing tumor cells (and non-reactive with a range of other tissues and cell types). Example 2 disclosed the results of an experiment conducted by Alonso in treating Melanie Brown, a patient with neurofibrosarcoma. Adult spleen cells were sensitized with cells from Brown's tumor. The resulting hybridoma secreted monoclonal antibodies, which reacted with a 221 KiloDalton tumor surface antigen. The spleen line (AS-151), the lymphoblast fusion line (BM-95), and the hybridoma (HB983) were deposited with the American Type Culture Collection in September of 1998. The antibody from the hybridoma line was deposited with the Food and Drug Administration.²

The examiner rejected claim 92 as lacking adequate written descriptive support for the broad genus of antibodies encompassed by the claim language.

² Alonso infused Brown with 100 mg of the antibody, and cancerous lesions in her lungs were cleared within twenty-four hours. In addition, Brown's brain tumor became necrotic within seven days, and she experienced a one-month regression of her cancer.

Applicant is reminded that the disclosure only describes the preparation of a single Mab produced by the hybridoma cell line HB983. However, the claims are directed toward a much larger genus of molecules (i.e., Mabs that bind to a neurofibrosarcoma), not a specific Mab identified by the deposited hybridoma. . . . The crux of the rejection is whether or not applicant has provided sufficient support for the broadly claimed genus of therapeutic antibodies. As set forth in the rejection, the skilled artisan would reasonably conclude that applicant was clearly not in possession of the claimed genus of compounds. Applicant should direct the claim language toward the only described embodiment (e.g., a Mab produced by hybridoma HB983).

The Board affirmed the rejection, agreeing that Alonso had not adequately described the claimed invention because the "single antibody described in the Specification is insufficiently representative to provide adequate written descriptive support for the genus of antibodies required to practice the claimed invention." Decision, slip op. at 7.

II. DISCUSSION

Whether an applicant has complied with the written description requirement is a finding of fact, to be analyzed from the perspective of one of ordinary skill in the art as of the date of the filing of the application. Regents of the Univ. of Cal. v. Eli Lilly & Co., 119 F.3d 1559, 1566 (Fed. Cir. 1997); Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563 (Fed. Cir. 1991). This Court reviews the Board's factual determinations under a substantial evidence standard. In re Gartside, 203 F.3d 1305, 1316 (Fed. Cir. 2000). "Substantial evidence" is relevant evidence that "a reasonable mind might accept as adequate to support a conclusion." Id. at 1312 (citation omitted). In making the assessment, we examine "the record as a whole, taking into account evidence that both justifies and detracts from an agency's decision." Id. That a fact finder could draw "two inconsistent conclusions from the evidence does not prevent an administrative agency's finding from being supported by substantial evidence." Id. (citation omitted). Rather,

the Board's decision must be affirmed if any "reasonable fact finder could have arrived at the [same] decision." Id.

The written description requirement of 35 U.S.C. § 112, ¶ 1, is straightforward: "The specification shall contain a written description of the invention" To satisfy this requirement, the specification must describe the invention in sufficient detail so "that one skilled in the art can clearly conclude that the inventor invented the claimed invention as of the filing date sought." Lockwood v. Am. Airlines, Inc., 107 F.3d 1565, 1572 (Fed. Cir. 1997); see also LizardTech, Inc. v. Earth Res. Mapping, Inc., 424 F.3d 1336, 1345 (Fed. Cir. 2005); Eiselstein v. Frank, 52 F.3d 1035, 1039 (Fed. Cir. 1995).

The requirement "serves a teaching function, as a 'guid pro quo' in which the public is given 'meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time.'" Univ. of Rochester v. G.D. Searle & Co., Inc., 358 F.3d 916, 922 (Fed. Cir. 2004) (quoting Enzo Biochem, Inc. v. GenProbe Inc., 323 F.3d 956, 970 (Fed. Cir. 2002)).³ The Board framed the issue raised by the '749 Application as follows.

[W]hether the single monoclonal antibody described in the Specification is representative of the genus of monoclonal antibodies required to practice the claimed treatment method. That, in turn, depends on whether or not the antibodies (and the antigens they bind) would have been expected to vary substantially within the genus. The greater the variation in the genus, the less representative any particular antibody would be.

Decision, slip op. at 6.

³ The requirement is rigorous, but not exhaustive: "[I]t is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention." LizardTech, 424 F.3d at 1345.

The Board properly characterized the relevant genus as the “genus of antibodies specific to neurofibrosarcoma cells.” Id. A genus can be described by disclosing: (1) a representative number of species in that genus; or (2) its “relevant identifying characteristics,” such as “complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.” Enzo, 323 F.3d at 964.

Relying principally on two scientific articles, including one authored by Alonso himself, the Board determined that

[t]here is ample evidence of record that the specificities of antibodies falling within the scope of the genus (and the structures of the antigens they bind) would be expected to vary substantially. For example, Osband⁴ provides evidence of a recognition in the art that considerable antigenic “heterogeneity of tumors both between patients and metastatic sites within a single patient” is to be expected. In addition, an article authored by [Alonso]⁵ acknowledges that “[t]he efficacy of antibody therapy is thought to be related to tumor burden as well as to idiotypic change in the original tumor.” This acknowledged heterogeneity is reflected in the goal of the claimed method - to raise customized antibodies to possibly unique antigens on a particular patient's tumor.

Finally, as discussed above, for purposes of satisfying the written description requirement, it is not enough merely to disclose a method of making and identifying compounds capable of being used to practice the claimed invention. That is, it is not enough to describe[] the procedure for making a human-human hybridoma from neurofibrosarcoma, and teach how to determine whether a given antibody, specific to a patient's neurofibrosarcoma, will function in the claimed method. We find that the single antibody described in the Specification is insufficiently representative to provide adequate written descriptive support for the genus of antibodies required to practice the claimed invention.

⁴ M.E. Osband and S. Ross, Problems in the Investigational Study and Clinical Use of Cancer Immunotherapy, 11 Immunology Today 193-95 (1990).

⁵ Kenneth Alonso, Human-Human Monoclonal Antibody Directed Against Tumor Surface Antigen in the Treatment of Human Malignancy, 14 American Journal of Clinical Oncology 463-71 (1991).

Decision, slip op. at 6-7 (internal quotation marks and citations omitted, footnotes supplemented).

The Board's conclusion is supported by substantial evidence. The articles relied upon by the Board confirm the hypothesis that the antibodies required to perform Alonso's claimed method vary substantially in their composition. We have previously held in a similar context that "a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from species other than those specifically enumerated." Noelle v. Lederman, 355 F.3d 1343, 1350 (Fed. Cir. 2004).

In another similar case, we evaluated claims directed to a method of determining whether a drug could selectively inhibit the activity of COX-2, a cyclooxygenase thought to be responsible for inflammation associated with arthritis. See Rochester, 358 F.3d at 917-18. One of the claims at issue was directed to "a method for selectively inhibiting [COX-2] activity in a human host, comprising administering a non-steroidal compound that selectively inhibits activity of the [COX-2] gene product to a human host in need of such treatment." Id. at 918. We found that the specification lacked written descriptive support, agreeing with the district court that

it is clear from reading the patent that one critical aspect of the method - a compound that selectively inhibits [COX-2] activity - was hypothetical, for it is clear that the inventors had neither possession nor knowledge of such a compound. . . . [T]he claimed method depends upon finding a compound that selectively inhibits [COX-2] activity. Without such a compound, it is impossible to practice the claimed method of treatment.

Id. at 926. We further noted that the specification contained "no disclosure of any method for making even a single 'non-steroidal compound that selectively inhibits

activity of the [COX-2] gene product,” and failed to “steer the skilled practitioner toward compounds that can be used to carry out the claimed methods.” Id. at 928, 929.

Alonso attempts to distinguish his claimed invention from Rochester by emphasizing that he reduced his method to practice and identified the resulting compound. We are not persuaded by the distinction. “[P]roof of a reduction to practice, absent an adequate description in the specification of what is reduced to practice, does not serve to describe or identify the invention for purposes of [the written description requirement].” Enzo, 323 F.3d at 969.⁶ Moreover, while it is true that Rochester disclosed no compounds that worked with the claimed method, the one compound disclosed by Alonso cannot be said to be representative of a densely populated genus.

In Rochester, we reasoned that while the specification

describes what can be done with any compounds that may potentially be identified through those assays, including formulation into pharmaceuticals, routes of administration, estimation of effective dosage, and suitable dosage forms . . . the '850 patent does not disclose just which peptides, polynucleotides, and small organic molecules have the desired characteristics of selectively inhibiting [COX-2]. Without such disclosure the claimed methods cannot be said to have been described.

Rochester, 358 F.3d at 927 (internal citation and quotation marks omitted). We additionally found that Rochester had failed to present any evidence that one skilled in

⁶ The reduction-to-practice argument also implicates § 112’s enablement requirement. The Board reversed the examiner’s rejection of claim 92 for lack of enablement. See Decision, slip op. at 12. Alonso argues that the Board’s findings as to sufficiency of description and enablement are at odds with one another. It is true that the written description and enablement requirements “usually rise and fall together. That is, a recitation of how to make and use the invention across the full breadth of the claim is ordinarily sufficient to demonstrate that the inventor possesses the full scope of the invention, and vice versa.” LizardTech, 424 F.3d at 1345. However, we have been clear that “[a]lthough the legal criteria of enablement and written description are related and are often met by the same disclosure, they serve discrete legal requirements.” Capon v. Eshhar, 418 F.3d 1349, 1360 (Fed. Cir. 2005). “[A]n invention may be enabled even though it has not been described.” Rochester, 358 F.3d at 921.

the art would have been able to isolate and identify any given compound based on Rochester's "vague functional description" Id. at 928. Even more recently, we held that the written disclosure requirement was not met where the claims at issue covered a broad "genus of recombinant plasmids that contain coding sequences for DNA polymerase . . . from any bacterial source, [but] the narrow specifications of the [relevant patents] only disclose[d] the . . . gene coding sequence from one bacterial source" Carnegie Mellon Univ. v. Hoffman-LaRoche Inc., 541 F.3d 1115, 1125 (Fed. Cir. 2008) (emphasis added).

The same is true here. The specification of the '749 Application does not characterize the antigens to which the monoclonal antibodies must bind; it discloses only the molecular weight of the one antigen identified in Example 2. This is clearly insufficient.⁷ The specification teaches nothing about the structure, epitope characterization, binding affinity, specificity, or pharmacological properties common to the large family of antibodies implicated by the method. While Alonso's claim is written as a method, the antibodies themselves are described in purely structural language – "a monoclonal antibody idiotypic to the neurofibrosarcoma of said human." This sparse description of antibody structure in the claim stands in stark contrast to the detailed method of making the antibodies found in the specification.

The Eli Lilly decision is also instructive. In Eli Lilly, the University of California laid claim to all vertebrate insulin cDNAs, including the human insulin cDNA, although it had identified only the cDNA for rat insulin. See Eli Lilly, 119 F.3d at 1567.⁸ We ruled

⁷ It bears mentioning that the examiner encouraged Alonso to amend his claims to cover only the MAB produced by the identified hybridoma.

⁸ The claims at issue in Eli Lilly were directed to "a recombinant plasmid

that the written description requirement was not met because “a description of rat insulin cDNA is not a description of the broad classes of vertebrate or mammalian insulin cDNA.” Id. at 1568. As in Eli Lilly, the specification of the '749 Application contains information about only one compound.⁹

Apart from the representative number of species test applied by the Board, we have found adequate written descriptive support for a claimed invention where the disclosure specifies “relevant identifying characteristics,” such as “complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.” Enzo, 323 F.3d at 964 (emphasis in original). Alonso argues that there is a well-known correlation between the structure and function of the neurofibrosarcoma-specific antibodies generated by his disclosed treatment method. He maintains that the members of the genus of antibodies directed to a particular patient's tumor share the same function - they each bind to a patient's neurofibrosarcoma, thereby bolstering the patient's immune mechanism and stimulating an attack on the tumor cells. As for structure, Alonso argues that because monoclonal antibodies are secreted from a hybridoma made from a particular neurofibrosarcoma,

replicable in [a] procaryotic host containing within its nucleotide sequence a subsequence having the structure of the reverse transcript of an mRNA of a *vertebrate*, which mRNA encodes insulin.” Eli Lilly, 119 F.3d at 1563 (emphasis in original).

⁹ Alonso cites In re Herschler, 591 F.2d 693 (CCPA 1979), where our predecessor court held that the disclosure of a single corticosteroid was sufficient to describe the genus of physiologically active steroids that could be used in practicing the claimed invention. The court based its decision on the fact that the class of implicated compounds was “chemically quite similar.” Id. at 701. Alonso argues that the same is true with respect to the antibodies generated by a patient's specific neurofibrosarcoma. However, he points to no evidence in the record corroborating his “similarity” thesis.

the antibodies are necessarily specific. He further argues that there is a “well-known correlation between human antibody structure and antibody function.”

Alonso did not raise this structure-function correlation argument in the proceedings before the Board. “Failure to advance legal theories before the [B]oard constitutes a failure to ‘make a complete presentation of the issues,’ and permitting a party to raise those theories for the first time [after the agency has rendered its final decision] would be both inefficient and ‘wasteful of administrative and judicial resources.’” Boston Scientific Scimed, Inc. v. Medtronic Vascular, Inc., 497 F.3d 1293, 1298 (Fed. Cir. 2007). Accordingly, we will not consider this newly minted argument on appeal.¹⁰

III. CONCLUSION

For the aforementioned reasons, the decision of the Board is affirmed.

AFFIRMED.

¹⁰ Even were we tempted to consider the argument, Alonso would not be entitled to relief. In Noelle, the applicant claimed a human monoclonal antibody (or fragment thereof) secreted from a particular hybridoma that binds to an antigen expressed on activated T-cells. The application did not, however, disclose any structural information about the human antigen. Noelle, 355 F.3d at 1345-46. It described only the mouse antigen. We concluded that the function-structure correlation test was not met.

If [the applicant] had sufficiently described the human form of CD40CR antigen, he could have claimed its antibody by simply stating its binding affinity for the “fully characterized” antigen. [The applicant] did not describe human CD40CR antigen. Therefore, [the applicant] attempted to define an unknown by its binding affinity to another unknown.

Noelle, 355 F.3d at 1349. As Alonso has not pointed to any structure for his claimed antibodies, there is no structure to which he may correlate the function of his claimed antibodies.

Enzo Biochem Inc. v. Gen-Probe Inc.

U.S. Court of Appeals
Federal Circuit

No. 01-1230

Decided July 15, 2002

PATENTS

[1] Patentability/Validity — Specification
— Written description (§ 115.1103)

Functional description of genetic material may be sufficient to satisfy written description requirement of 35 U.S.C. § 112, since requirement can be met by showing that invention is completely disclosed of sufficiently detailed, relevant identifying characteristics, including functional characteristics when coupled with known or disclosed correlation between function and structure.

[2] Patentability/Validity — Specification
— Written description (§ 115.1103)

Reference in specification to deposit of biological material in public depository, which makes its contents accessible to public when it is not otherwise available in written form, constitutes adequate description of deposited material sufficient to comply with the written description requirement of 35 U.S.C. § 112; in present case, in which patent in suit is directed to nucleic acid probes, reference in specification to deposits of nucleotide sequences describe those sequences sufficiently to public for purposes of meeting written description requirement.

[3] Patentability/Validity — Specification
— Written description (§ 115.1103)

Written description requirement for generic claims is not necessarily met as matter of law merely because claim language is repeated verbatim in specification, since, even if claim is supported by specification, language of specification must, to extent possible, describe claimed invention so that one skilled in art can recognize what is claimed, and appearance of mere indistinct words in specification or claim does not satisfy that requirement; specification does not necessarily describe invention by indicating that applicant "possessed" invention as of desired filing date, since ensuring that applicant had possession of invention is one

purpose of description requirement, but possession alone is not always sufficient to satisfy that requirement.

[4] Patentability/Validity — Date of invention
— Reduction to practice
(§ 115.0405)Patentability/Validity — Specification —
Written description (§ 115.1103)

Proof of reduction to practice, absent adequate description in specification of what is reduced to practice, does not satisfy written description requirement of 35 U.S.C. § 112, since proof of reduction to practice may show priority of invention, but it does not by itself provide written description in patent's specification; in present case, patentee's disclosure of actual reduction to practice is not "safe haven" by which it has demonstrated compliance with description requirement.

Particular patents — Chemical —
Nucleic acid probes

4,900,659, Lo and Yang, nucleotide sequence composition and method for detection of *Neisseria gonorrhoeae* and method for screening for a nucleotide sequence that is specific for a genetically distinct group, summary judgment of invalidity reversed on rehearing.

Appeal from the U.S. District Court for the Southern District of New York, Hellerstein, J.

Action by Enzo Biochem Inc. against Gen-Probe Inc., Chugai Pharma U.S.A. Inc., Chugai Pharmaceutical Co. Ltd., Biomerieux Inc., Becton Dickinson and Co., and Biomerieux SA for patent infringement. Summary judgment of patent invalidity was affirmed on appeal in panel opinion issued April 2, 2002 (62 USPQ2d 1289). On plaintiff-appellant's petition for rehearing, case was referred to merits panel that heard appeal. Petition granted; prior decision vacated; district court's grant of summary judgment reversed and remanded.

Richard L. Delucia, Charles A. Weiss, and Bradley S. Corsello, of Kenyon & Kenyon, New York, N.Y., for plaintiff-appellant.

William F. Lee and William G. McElwain, of Hale and Dorr, Boston, Mass., for defendant-appellee Gen-Probe Inc.

11th Circuit, 28 U.S.C. § 1631; see *Johnson v. Colt Indus. Operating U.S. 800, 818* [7 USPQ2d 1109] finding that upon the Federal Circuit determination that it did not have its sole choice was to dismiss the *isler* it to a court of appeals with *); Schwartzkopf Dev. Corp. v. Ti-* 800 F.2d 240, 245, 231 USPQ 1, Cir. 1986) (transferring case to court under transfer statute upon the jurisdiction did not lie with the court).

II. CONCLUSION

diction for this case as originally circuit was predicated on a patent it counterclaim. However, as a re-Supreme Court's intervening decisions, we do not have appellate ju-ver the cases. As a result, we do he issues appealed and order this red to the Eleventh Circuit.

TRANSFERRED.

COSTS

Robert J. Gunther Jr., Jeffrey A. Tochner, and Kurt M. Rogers, of Latham & Watkins, New York, for defendants-appellees Chugai Pharma U.S.A. Inc. and Chugai Pharmaceutical Co. Ltd.

Daniel A. Bochnen and Joshua R. Rich, of McDonnell Bochnen Hulbert & Berghoff, Chicago, Ill., for defendant-appellee Biomerieux Inc.

Donald R. Ware and Barbara A. Fiocco, of Foley Hoag & Eliot, Boston, for defendant-appellee Becton Dickinson and Co.

Frank P. Porcelli, Robert E. Hillman, and Charles H. Sanders, of Fish & Richardson, Boston, for amicus curiae Fish & Richardson P.C.

Mark S. Davies, attorney, Robert D. McCallum Jr., assistant attorney general, and Scott R. McIntosh, attorney, U.S. Department of Justice, Washington, for amicus curiae United States in support of rehearing en banc.

Before Lourie, Dyk, and Prost, circuit judges.

Lourie, J.

ON PETITION FOR REHEARING

Enzo Biochem, Inc. petitions for rehearing of this appeal following our prior decision, reported at 285 F.3d 1013, 62 USPQ2d 1289 (Fed. Cir. 2002), in which we affirmed the decision of the United States District Court for the Southern District of New York. The district court had granted Gen-Probe Incorporated, Chugai Pharma U.S.A., Inc., Chugai Pharmaceutical Co., Ltd., Biomerieux, Inc., Biomerieux SA, and Becton Dickinson and Company's (collectively, "the defendants'") motion for summary judgment that claims 1-6 of U.S. Patent 4,900,659 are invalid for failure to meet the written description requirement of 35 U.S.C. § 112, ¶ 1. *Enzo Biochem, Inc. v. Gen-Probe Inc.*, No. 99 Civ. 4548 (S.D.N.Y. Apr. 4, 2001) (final order). Having considered Enzo's petition for rehearing and the defendants' response,¹ we have determined that our prior decision that a deposit may not satisfy the written description requirement was incorrect. We therefore grant Enzo's petition for rehearing, vacate the prior decision, and reverse the district court's grant

of summary judgment that Enzo's claims are invalid for failure to meet the written description requirement. Because genuine issues of material fact exist regarding satisfaction of the written description requirement, we remand.

BACKGROUND

Enzo is the assignee of the '659 patent, which is directed to nucleic acid probes that selectively hybridize to the genetic material of the bacteria that cause gonorrhea, *Neisseria gonorrhoeae*. *N. gonorrhoeae* reportedly has between eighty and ninety-three percent homology with *Neisseria meningitidis*. '659 patent, col. 2, ll. 61-64. Such a high degree of homology has made detection of *N. gonorrhoeae* difficult, as any probe capable of detecting *N. gonorrhoeae* may also show a positive result when only *N. meningitidis* is present. Enzo recognized the need for a chromosomal DNA probe specific for *N. gonorrhoeae*, and it derived three such sequences that preferentially hybridized to six common strains of *N. gonorrhoeae* over six common strains of *N. meningitidis*. *Id.* at col. 3, l. 49 to col. 4, l. 14; col. 4, ll. 45-50. The inventors believed that if the preferential hybridization ratio of *N. gonorrhoeae* to *N. meningitidis* were greater than about five to one, then the "discrete nucleotide sequence [would] hybridize to virtually all strains of *Neisseria gonorrhoeae* and to no strain of *Neisseria meningitidis*." *Id.* at col. 12, ll. 60-65. The three sequences that the inventors actually derived had a selective hybridization ratio of greater than fifty. *Id.* at col. 13, ll. 9-15. Enzo deposited those sequences in the form of a recombinant DNA molecule within an *E. coli* bacterial host at the American Type Culture Collection. *Id.* at col. 13, ll. 27-31.

Claim 1 is as follows:

1. A composition of matter that is specific for *Neisseria gonorrhoeae* comprising at least one nucleotide sequence for which the ratio of the amount of said sequence which hybridizes to chromosomal DNA of *Neisseria gonorrhoeae* to the amount of said sequence which hybridizes to chromosomal DNA of *Neisseria meningitidis* is greater than about five, said ratio being obtained by a method comprising the following steps;

(a) providing a radioactively labeled form of said nucleotide sequence;

¹ Amicus curiae briefs were filed by the United States Patent and Trademark Office and Fish & Richardson P.C.

63 USPQ2d

63 USPQ2d

Enzo Biochem Inc. v. Gen-Probe Inc.

1611

ment that Enzo's claims are to meet the written description. Because genuine issues of regarding satisfaction of the a requirement, we remand.

BACKGROUND

significance of the '659 patent, to nucleic acid probes that are to the genetic material of cause gonorrhea, *Neisseria gonorrhoeae* reportedly has no ninety-three percent homology to *Neisseria meningitidis*. '659 is 11-64. Such a high degree of similarity of *N. gonorrhoeae* may also show a position only *N. meningitidis* is recognized the need for a chromosome specific for *N. gonorrhoeae* three such sequences hybridized to six common *gonorrhoeae* over six common *meningitidis*. *Id.* at col. 3, l. 49 to 50, ll. 45-50. The inventors believed that hybridization reactions to *N. meningitidis* were not five to one, then the "discrepancy [would] hybridize strains of *Neisseria meningitidis*, ll. 60-65. The three sequences actually derived hybridization ratio of greater than 13, ll. 9-15. Enzo deposits the material in the form of a recombinant within an *E. coli* bacterial strain Type Culture Collection. 17-31.

allows:
on of matter that is specific *gonorrhoeae* comprising a nucleotide sequence for which the amount of said sequence hybridizes to chromosomal DNA *gonorrhoeae* to the amount of sequence which hybridizes to the DNA of *Neisseria meningitidis* more than about five, said ratio determined by a method comprising the following steps;

g a radioactively labeled nucleotide sequence;

(b) providing a serial dilution series of purified chromosomal DNA from each of the *N. gonorrhoeae* strains; (1) ATCC 53420, (2) ATCC 53421, (3) ATCC 53422, (4) ATCC 53423, (5) ATCC 53424, (6) ATCC 53425, and forming test dots from each of said dilution series on a matrix;

(c) providing a serial dilution series of purified nucleotide sequences from each of the *N. meningitidis* strains: (1) ATCC 53414, (2) ATCC 53415, (3) ATCC 53416, (4) ATCC 53417, (5) ATCC 53418, (6) ATCC 53419, and forming test dots from each of said dilution series on a matrix;

(d) hybridizing equal portions of the labeled nucleotide sequences to the matrix provided in step (b) and (c), respectively; wherein the hybridization is conducted in a solution having a salt concentration of 2X SSC at (i) 65°C. in cases in which the sequence has greater than 50 base pairs or (ii) at Tm (°C.) minus 30°C. in cases in which the sequence has less than 50 base pairs, wherein Tm is the denaturation temperature of the sequence;

(e) quantifying the labeled nucleotide sequence hybridized in step (d) to each test dot;

(f) subtracting from the data of step (e) an averaged amount of radioactivity attributable to background to obtain a corrected amount of hybridized radioactivity at each test dot;

(g) normalizing the data of step (f) by multiplying the amount of corrected radioactivity at each test dot by a factor which adjusts the amount of radioactivity to equal amounts of chromosomal DNA at each test dot;

(h) selecting two normalized values that are most nearly the same and that correspond to adjacent members of the dilution series for each of the above strains of *N. gonorrhoeae* and obtaining the average of the selected values;

(i) selecting two normalized values that are most nearly the same and that correspond to adjacent members of the dilution series for each of the above strains of *N. meningitidis* and obtaining the average of the selected values;

(j) dividing the lowest average obtained in step (h) by the highest average obtained in step (i) to obtain said ratio.

Id. at col. 27, l. 29 to col. 28, l. 27 (emphasis added). Claims 2 and 3 depend from claim 1 and further limit the hybridization ratio to greater than about twenty-five and fifty, respectively. *Id.* at col. 2, ll. 27-30. Claim 4 is directed to the three deposited sequences (referred by their accession numbers) and variants thereof as follows:

4. The composition of claim 1 wherein said nucleotide sequences are selected from the group consisting of:

a. the *Neisseria gonorrhoeae* [sic] DNA insert of ATCC 53409, ATCC 53410 and ATCC 53411, and discrete nucleotide subsequences thereof,

b. mutated discrete nucleotide sequences of any of the foregoing inserts that are within said hybridization ratio and subsequences thereof; and

c. mixtures thereof.
Id. at col. 28, ll. 31-39. Claim 5 is directed to an assay for detection of *N. gonorrhoeae* using the composition of claim 1. *Id.* at ll. 40-46. Claim 6 further limits the method of claim 5 to the nucleotide sequences that Enzo deposited (i.e., those in claim 4) and variants thereof. *Id.* at ll. 47-56.

Enzo sued the defendants for infringement of the '659 patent, and the defendants moved for summary judgment that the claims were invalid for failure to meet the written description requirement of 35 U.S.C. § 112, ¶ 1. The district court, in oral remarks from the bench, granted that motion. *Tr. of Hr'g* at 42, *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, No. 99-CV-4548 (S.D.N.Y. Jan. 24, 2001). It concluded that the claimed composition of matter was defined only by its biological activity or function, viz., the ability to hybridize to *N. gonorrhoeae* in a ratio of better than about five with respect to *N. meningitidis*, which it was held was insufficient to satisfy the § 112, ¶ 1 requirement set forth in this court's holdings in *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993), and *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991). *Tr. of Hr'g* at 28. The court rejected

Enzo's argument that the reference in the specification to the deposits of biological materials in a public depository inherently disclosed that the inventors were in possession of the claimed sequences. *Id.* at 35. It distinguished this court's precedents concerning deposits as relating to the enablement requirement of § 112, ¶ 1. *Id.* at 38-40. Enzo appealed to this court; we have jurisdiction pursuant to 28 U.S.C. § 1295 (a)(1).

DISCUSSION

Summary judgment is appropriate when there is no genuine issue of material fact and the moving party is entitled to judgment as a matter of law. Fed. R. Civ. P. 56(c); *Anderson v. Liberty Lobby, Inc.*, 477 U.S. 242, 247-48 (1986). On motion for summary judgment, the court views the evidence and any disputed factual issues in the light most favorable to the party opposing the motion. *Matsushita Elec. Indus. Co. v. Zenith Radio Corp.*, 475 U.S. 574, 587 (1986). A patent is presumed to be valid. 35 U.S.C. § 282 (1994), and this presumption can be overcome only by facts supported by clear and convincing evidence to the contrary, *see, e.g., WMS Gaming, Inc. v. Int'l Game Tech.*, 184 F.3d 1339, 1355, 51 USPQ2d 1385, 1396-97 (Fed. Cir. 1999). Compliance with the written description requirement is a question of fact. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991).

Enzo argues that the testimony of its expert, Dr. Wetmur, raised a genuine factual issue whether the reference to the deposits inherently described the claimed nucleotide sequences. Enzo also argues that its description of the binding affinity of the claimed nucleotide sequences satisfies the requirement set forth in the Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement, 66 Fed. Reg. 1099 (Jan. 5, 2001) ("Guidelines"). Enzo asserts that the court erred in not evaluating the patentability of the claims separately, pointing out that claims 4 and 6 are directed to the three deposited sequences and variations and mixtures thereof. Enzo further asserts that the claims *per se* meet the written description requirement because they appear *in ipso verbi* in the written description. Enzo also argues that this court's articulation of the written description requirement for genetic material in *Eli Lilly* should not apply to this case because Enzo reduced the invention to

practice and deposited the derived biological materials, thereby demonstrating its "possession" of the invention.

The defendants respond that the district court properly granted summary judgment because the patent described the claimed nucleotide sequences only by their function, which they state is insufficient to meet the requirements of § 112, ¶ 1 as a matter of law, even as to the narrower claims directed to the deposited materials. The defendants also assert that Dr. Wetmur's opinion that the deposited genetic materials could have been sequenced did not cure the actual failure of the inventors to identify them by some distinguishing characteristic, such as their structure. Moreover, the defendants point out that claims 4 and 6, which are directed to the deposited materials, each cover a broad genus of nucleic acids. The defendants also urge that *in ipso verbi* support for the claims in the specification does not *per se* establish compliance with the written description requirement. Finally, the defendants assert that the district court did not err in its determination that Enzo's "possession" of three nucleotide sequences that it reduced to practice and deposited nevertheless did not satisfy the written description requirement of § 112, ¶ 1.

The written description requirement of § 112, ¶ 1 is set forth as follows:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

35 U.S.C. § 112, ¶ 1 (1994) (emphasis added). We have interpreted that section as requiring a "written description" of an invention separate from enablement. *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1117 (recognizing the severability of the "written description" and "enablement" provisions of § 112, ¶ 1). Compliance with the written description requirement is essentially a fact-based inquiry that will "necessarily vary depending on the nature of the invention claimed." *Id.* (citing *In re DiLeone*, 436 F.2d 1404, 1405, 168 USPQ 592, 593 (CCPA 1971)). We have also previously considered the written description requirement

63 USPQ2d

deposited the derived biological
by demonstrating its "possession."

ants responded that the district granted summary judgment because it described the claimed nucleic acids only by their function, which is sufficient to meet the requirements of 35 U.S.C. § 101, ¶ 1 as a matter of law, even if the claims directed to the details of the structure. The defendants also asserted that the district court erred in its opinion that the deposited samples could have been sequenced to ascertain the actual sequence of the inventors' nucleic acids. The district court responded by pointing out that claims 4 and 6, which recited the deposited materials, also recited the genus of nucleic acids. The district court urged that *in ipsa verbi* suffices to distinguish the deposited materials from prior art. The district court also found that the defendants' compliance with the written description requirement. Finally, the district court found that the district court did not determine that Enzo's "possession" of the nucleotide sequences that it recited and deposited nevertheless satisfied the written description requirement.

A description requirement of
it forth as follows:

tion shall contain a written description of the invention, and of the manner of making and using it, in clear, concise, and exact terms as any person skilled in the art to which it relates or with which it is most connected, to make and use the same, shall set forth the best mode contemplated by the inventor of carrying out the same.

2, ¶ 1 (1994) (emphasis added).
reted that section as requiring a
ption" of an invention separate
nt. *Vas-Cath*, 935 F.2d at 1563,
: 1117 (recognizing the sever-
"written description" and "en-
visions of § 112, ¶ 1). Compli-
written description requirement
a fact-based inquiry that will
vary depending on the nature of
claimed." *Id.* (citing *In re Dille-*
1404, 1405, 168 USPQ 592,
711)). We have also previously
written description requirement

63 USPO2d

Enzo Biochem Inc. v. Gen-Probe Inc.

1613

as applied to certain biotechnology patents, in which a gene material has been defined only by a statement of function or result, and have held that such a statement alone did not adequately describe the claimed invention. *Eli Lilly, 119 F.3d at 1568, 43 USPQ2d at 1406*. In *Eli Lilly*, we concluded that a claim to a microorganism containing a human insulin cDNA was not adequately described by a statement that the invention included human insulin cDNA. *Id.* at 1567, 43 USPQ2d at 1405. The recitation of the term human insulin cDNA conveyed no distinguishing information about the identity of the claimed DNA sequence, such as its relevant structural or physical characteristics. *Id.* We stated that an adequate written description of genetic material "requires a precise definition, such as by structure, formula, chemical name, or physical properties," not a mere wish or plan for obtaining the claimed chemical invention," and that none of those descriptions appeared in that patent. *Id.* at 1566, 43 USPQ2d at 1404 (quoting *Fiers, 984 F.2d at 1171, 25 USPQ2d at 606*). The Specification in the *Eli Lilly* case thus did not show that the inventors had possession of human insulin cDNA.

[1] It is not correct, however, that all functional descriptions of genetic material fail to meet the written description requirement. The PTO has issued Guidelines governing its internal practice for addressing that issue. The Guidelines, like the Manual of Patent Examining Procedure ("MPEP"), are not binding on this court, but may be given judicial notice to the extent they do not conflict with the statute. See *Molins PLC v. Vtextron, Inc.*, 48 F.3d 1172, 1180 n.13, 33 USPQ2d 1823, 1828 n.10 (Fed. Cir. 1995). In its Guidelines, the PTO has determined that the written description requirement can be met by "show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." Guidelines, 66 Fed. Reg. at 1106 (emphasis added). For example, the PTO would find compliance with § 112, ¶ 1, for a claim to an "isolated antibody capable of binding to antigen X," notwithstanding the functional definition of the antibody, in light of "the well defined structural characteristics

for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature." Synopsis of Application of Written Description Guidelines, at 60, available at <http://www.uspto.gov/web/patents/guides.htm> ("Application of Guidelines"). Thus, under the Guidelines, the written description requirement would be met for all of the claims of the '659 patent if the functional characteristic of preferential binding to *N. gonorrhoeae* over *N. meningitidis* were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed. We are persuaded by the Guidelines on this point and adopt the PTO's applicable standard for determining compliance with the written description requirement.

Applying those principles, we first inquire whether Enzo's deposits of the claimed nucleotide sequences of claims 4 and 6 may constitute an adequate description of those sequences. Secondly, we will consider whether the description requirement is met for all of the claims on the basis of the functional ability of the claimed nucleotide sequences to hybridize to strains of *N. gonorrhoeae* that are accessible by deposit.

[2] As to the first question, Enzo asserts that the claimed sequences are inherently described by reference to deposits of three sequences that are within the scope of its claims. Whether reference to a deposit of a nucleotide sequence may adequately describe that sequence is an issue of first impression in this court. In light of the history of biological deposits for patent purposes, the goals of the patent law, and the practical difficulties of describing unique biological materials in a written description, we hold that reference in the specification to a deposit in a public depository, which makes its contents accessible to the public when it is not otherwise available in written form, constitutes an adequate description of the deposited material sufficient to comply with the written description requirement of § 112, ¶ 1.

The practice of depositing biological material arose primarily to satisfy the enablement requirement of § 112, ¶ 1. For example, in *In re Argoudelis*, the patent application claimed antibiotic compounds that were produced by a microorganism. 434 F.2d 1390, 1390, 168 USPQ 99, 100 (CCPA 1970). The applicants deposited the microorganism because they

could not "sufficiently disclose by written word how to obtain the microorganism starting material from nature." *Id.* at 1392, 168 USPQ at 102. By making the biological material accessible to the public, they enabled the public to make and use the claimed antibiotics. *Id.* at 1393, 168 USPQ at 102-03. In *Amgen*, we noted the relevance of deposit practice to satisfaction of the enablement requirement but rejected the defendants' argument that a deposit was necessary in that case to satisfy the best mode requirement of § 112, ¶ 1. *See* 927 F.2d at 1210, 18 USPQ2d at 1024; *see also In re Lundak*, 773 F.2d 1216, 1217, 227 USPQ 90, 92 (Fed. Cir. 1985) (discussing deposit practice primarily in relation to an enablement rejection and noting that "[a]n accession number and deposit date add nothing to the written description of the invention" in the context of proven availability of a cell line prior to filing date).

Recognizing the importance of biological deposits to patent practice, the PTO has promulgated rules to address the procedural requirements relating to such deposits, but it has declined to expressly correlate substantive requirements relating to deposits with particular statutory requirements. *See* Deposit of Biological Materials for Patent Purposes, 53 Fed. Reg. 39,420, 39,425 (Oct. 6, 1988) (notice of proposed rules) (codified at 37 C.F.R. Part 1) ("The rules are not intended to address which requirements of 35 U.S.C. 112 may be met by the making of deposits."). The Office does offer guidance, however, in determining when a deposit may be necessary, such as "[w]here the invention involves a biological material and words alone cannot sufficiently describe how to make and use the invention in a reproducible manner." MPEP § 2402 (8th ed. Aug. 2001). The PTO has also issued a regulation stating when a deposit is not necessary, *i.e.*, "if it is known and readily available to the public or can be made or isolated without undue experimentation." 37 C.F.R. § 1.802(b) (2001). Inventions that cannot reasonably be enabled by a description in written form in the specification, but that otherwise meet the requirements for patent protection, may be described in surrogate form by a deposit that is incorporated by reference into the specification. While deposit in a public depository most often has pertained to satisfaction of the enablement requirement, we have concluded that reference in the specification to a deposit

may also satisfy the written description requirement with respect to a claimed material.

In this case, Enzo's deposits were incorporated by reference in the specification. A person of skill in the art, reading the accession numbers in the patent specification, can obtain the claimed sequences from the ATCC depository by following the appropriate techniques to excise the nucleotide sequences from the deposited organisms containing those sequences. '659 patent, col. 13, ll. 27-36. The sequences are thus accessible from the disclosure in the specification. Although the structures of those sequences, *i.e.*, the exact nucleotide base pairs, are not expressly set forth in the specification, those structures may not have been reasonably obtainable and in any event were not known to Enzo when it filed its application in 1986. *See* '659 patent, col. 3, ll. 40-46 (noting severe time constraints in sequencing DNA). We therefore agree with Enzo that reference in the specification to deposits of nucleotide sequences describe those sequences sufficiently to the public for purposes of meeting the written description requirement.

As the defendants point out, however, Enzo's claims 4 and 6 are not limited to the deposited sequences. Claim 4 is directed to nucleotide sequences that are selected from the group consisting of the three deposited sequences, "discrete nucleotide subsequences thereof . . . mutated discrete nucleotide sequences of any of the foregoing inserts that are within said hybridization ratio and subsequences thereof," and . . . mixtures thereof." '659 patent, col. 28, ll. 31-39. Claim 6 is also similarly directed to the three deposited sequences and subsequences and mutated variations thereof. *Id.* at ll. 47-56. The specification defines a subsequence non-specifically as a nucleotide sequence "greater than about 12 nucleotides." '659 patent, col. 3, ll. 29-30. As the deposited sequences are about 850, 850, and 1300 nucleotides long, *id.* at col. 13, ll. 47-49, there are at least hundreds of subsequences of the deposited sequences, an unknown number of which might also meet the claimed hybridization ratio. Moreover, Enzo's expert, Dr. Wetmur, stated that "astronomical" numbers of mutated variations of the deposited sequences also fall within the scope of those claims, and that such broad claim scope is necessary to adequately protect Enzo's invention from copyists who could otherwise

tify the written description re-
 spect to a claimed material.
 e, Enzo's deposits were incorpo-
 rate in the specification. A per-
 son in the art, reading the accession
 re patent specification, can obtain
 sequences from the ATCC deposi-
 ting the appropriate techniques
 : nucleotide sequences from the
 rganisms containing those se-
 9 patent, col. 13, ll. 27-36. The
 e thus accessible from the disclo-
 sure specification. Although the struc-
 e sequences, i.e., the exact nucle-
 urs, are not expressly set forth in
 ition, those structures may not
 easonably obtainable and in any
 o known to Enzo when it filed its
 n 1986. See '659 patent, col. 3, ll.
 g severe time constraints in se-
 NA). We therefore agree with
 fference in the specification to de-
 leotide sequences describe those
 efficiently to the public for pur-
 suing the written description re-

endants point out, however, En-
 4 and 6 are not limited to the de-
 nences. Claim 4 is directed to
 equences that are selected from
 nisting of the three deposited se-
 leicrete nucleotide subsequences
 mutated discrete nucleotide se-
 ay of the foregoing inserts that
 aid hybridization ratio and subse-
 eoff[,] and . . . mixtures thereof."
 col. 28, ll. 31-39. Claim 6 is also
 ected to the three deposited se-
 subsequences and mutated varia-
 . *Id.* at ll. 47-56. The specification
 ibsequently non-specifically as a
 equence "greater than about 12
 "' 659 patent, col. 3, ll. 29-30. As
 d sequences are about 850, 850,
 eotide long, *id.* at col. 13, ll.
 : are at least hundreds of subse-
 e deposited sequences, an un-
 ber of which might also meet the
 ridization ratio. Moreover, Enzo's
 Wetmur, stated that "astronomi-
 s of mutated variations of the de-
 nences also fall within the scope of
 , and that such broad claim scope
 o adequately protect Enzo's in-
 n copyists who could otherwise

make a minor change to the sequence and
 thereby avoid infringement while still exploit-
 ing the benefits of Enzo's invention. The de-
 fendants assert that such breadth is fatal to the
 adequacy of the written description. On the
 other hand, because the deposited sequences
 are described by virtue of a reference to their
 having been deposited, it may well be that
 various subsequences, mutations, and mix-
 tures of those sequences are also described to
 one of skill in the art. We regard that question
 as an issue of fact that is best resolved on re-
 mand.² Because the district court's grant of
 summary judgment was based on its conclu-
 sion that Enzo's deposits could not satisfy the
 written description requirement as a matter of
 law, we reverse the district court's grant of
 summary judgment that claims 4 and 6 are in-
 valid for failure to meet the written descrip-
 tion requirement. On remand, the court should
 determine whether a person of skill in the art
 would glean from the written description, in-
 cluding information obtainable from the de-
 posits of the claimed sequences, subse-
 quences, mutated variants, and mixtures suffi-
 cient to demonstrate possession of the generic
 scope of the claims.

We next address the question whether the
 compositions of the broader genus claims 1-3
 and 5 are sufficiently described to meet the re-
 quirements of § 112, ¶ 1, on the basis of En-
 zo's deposits of three sequences. If those se-
 quences are representative of the scope of the
 genus claims, i.e., if they indicate that the pa-
 tencee has invented species sufficient to consti-
 tute the genera, they may be representative of
 the scope of those claims. See *In re Smythe*,
 480 F.2d 1376, 1383, 178 USPQ 279, 284-85
 (CCPA 1973) (discussing circumstances in
 which a species may be representative of and
 therefore descriptive of genus claims). Be-
 cause the district court concluded that the de-
 posited sequences were not themselves de-
 scribed, it did not determine whether that de-
 scription was representative of the genera in
 those claims. Such determination should be
 made on remand.

When we addressed a similar issue in *Eli
 Lilly*, we determined that a disclosure of the
 sequence of rat cDNA was not descriptive of

² We do not address the issue whether the
 breadth of the claim may implicate other va-
 lidity issues, such as enablement. Only written
 description is before us.

the broader invention consisting of mamma-
 lian and vertebrate cDNA, although it was a
 species falling within the scope of those
 claims. *Eli Lilly*, 119 F.3d at 1567-68, 43
 USPQ2d at 1405. In *Eli Lilly*, the specification
 and generic claims to all cDNAs encoding for
 vertebrate or mammalian insulin did not de-
 scribe the claimed genus because they did not
 set forth any common features possessed by
 members of the genus that distinguished them
 from others. *Id.* at 1568, 43 USPQ2d at 1405.
 Nor did the specification describe a sufficient
 number of species within the very broad ge-
 nus to indicate that the inventors had made a
 generic invention, i.e., that they had posses-
 sion of the breadth of the genus, as opposed
 to merely one or two such species. *Id.* The
 PTO has included a hypothetical example
 based on the facts of *Eli Lilly* in its Synopsis
 of Application of Written Description Guide-
 lines in which the description requirement is
 not met. See *Application of Guidelines*, Ex-
 ample 17, at 61-64. The PTO has also pro-
 vided a contrasting example of genus claims
 to nucleic acids based on their hybridization
 properties, and has determined that such
 claims may be adequately described if they
 hybridize under highly stringent conditions to
 known sequences because such conditions
 dictate that all species within the genus will be
 structurally similar. See *id.*, Example 9, at 35-
 37. Whether the disclosure provided by the
 three deposits in this case, coupled with the
 skill of the art, describes the genera of claims
 1-3 and 5 is a fact question the district court
 did not address. On remand, the district court
 should determine, consistently with the prece-
 dent of this court and the PTO's Guidelines,
 whether one skilled in the art would consider
 the subject matter of claims 1-3 and 5 to be
 adequately described, recognizing the signifi-
 cance of the deposits and the scope of the
 claims.

Enzo argues that all of the claims are ad-
 equately described on another basis, viz., by
 means of the disclosed correlation of the func-
 tion of hybridization with the bacterial DNA.
 In its petition for rehearing, Enzo states as at-
 torney argument that "[t]he description and
 claiming of biological materials by their affi-
 nity to other materials that are clearly identi-
 fied in the specification and claims (the particu-
 lar deposited strains of *N. gonorrhoeae* and *N.
 meningitidis*) inherently specifies structure,
 and is routine in this field." Claim 1 sets forth

the deposit numbers of six strains of *N. gonorrhoeae* to which the claimed nucleotide sequences preferentially hybridize, as well as the deposit numbers of six strains of *N. meningitidis* that are thereby distinguished. Again, as with the claimed nucleotide sequences, the sequences of the genomic DNA of those bacteria are not disclosed, perhaps because such sequencing would have been unduly burdensome at the time of Enzo's invention. '659 patent, col. 3, ll. 40-46 (noting that it would take 3,000 scientists one month to sequence the genome of one strain of *N. gonorrhoeae* and one strain of *N. meningitidis*). However, as those bacteria were deposited, their bacterial genome is accessible and, under our holding today, they are adequately described in the specification by their accession numbers. Because the claimed nucleotide sequences preferentially bind to the genomic DNA of the deposited strains of *N. gonorrhoeae* and have a complementary structural relationship with that DNA, those sequences, under the PTO Guidelines, may also be adequately described. Although the patent specification lacks description of the location along the bacterial DNA to which the claimed sequences bind, Enzo has at least raised a genuine issue of material fact as to whether a reasonable factfinder could conclude that the claimed sequences are described by their ability to hybridize to structures that, while not explicitly sequenced, are accessible to the public. Such hybridization to disclosed organisms may meet the PTO's Guidelines stating that functional claiming is permissible when the claimed material hybridizes to a disclosed substrate. That is a fact question. We therefore conclude that the district court erred in granting summary judgment that the claims are invalid for failure to meet the written description requirement. On remand, the court should consider whether one of skill in the art would find the generically claimed sequences described on the basis of Enzo's disclosure of the hybridization function and an accessible structure, consistent with the PTO Guidelines. If so, the written description requirement would be met.

[3] We next address Enzo's additional argument that the written description requirement for the generic claims is necessarily met as a matter of law because the claim language appears *in ipsius verbis* in the specification. We do not agree. Even if a claim is supported by

the specification, the language of the specification, to the extent possible, must describe the claimed invention so that one skilled in the art can recognize what is claimed. The appearance of mere indistinct words in a specification or a claim, even an original claim, does not necessarily satisfy that requirement. One may consider examples from the chemical arts. A description of an anti-inflammatory steroid, *i.e.*, a steroid (a generic structural term) described even in terms of its function of lessening inflammation of tissues fails to distinguish any steroid from others having the same activity or function. Similarly, the expression "an antibiotic penicillin" fails to distinguish a particular penicillin molecule from others possessing the same activity. A description of what a material does, rather than of what it is, usually does not suffice. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. The disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter purportedly described. *Id.*

In *Eli Lilly*, we were faced with a set of facts in which the words of the claim alone did not convey an adequate description of the invention. *Id.* at 1567, 43 USPQ2d at 1405. In such a situation, regardless whether the claim appears in the original specification and is thus supported by the specification as of the filing date, § 112, ¶ 1 is not necessarily met. See *Guidelines* at 1100 (noting *Eli Lilly's* clarification of the "original claim" doctrine in situations in which the name of the claimed material does not convey sufficient identifying information). If a purported description of an invention does not meet the requirements of the statute, the fact that it appears as an original claim or in the specification does not save it. A claim does not become more descriptive by its repetition, or its longevity.

Inasmuch as § 112, ¶ 1 requires such description, we are not persuaded by Enzo's argument that, because the specification indicated that Enzo "possessed" the claimed invention by reducing three sequences within the scope of the claims to practice, Enzo necessarily described the invention. It is true that in *Vas-Cath*, we stated: "The purpose of the 'written description' requirement is broader than to merely explain how to 'make and use'; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention." *Vas-Cath*, 935 F.2d

63 USPQ2d

63 USPQ2d

Enzo Biochem Inc. v. Gen-Probe Inc.

1617

tion, the language of the specific extent possible, must describe invention so that one skilled in the art can make and use the invention. The appearance of indistinct words in a specification, even an original claim, does not satisfy that requirement. One example from the chemical art is an anti-inflammatory steroid (a generic structural formula even in terms of its function). Inflammation of tissues fails to distinguish it from other steroids having the same function. Similarly, the ex-antibiotic penicillin fails to distinguish penicillin molecule from other antibiotics. A description of a material does, rather than does not, usually does not suffice. *Eli Lilly*, 1568, 43 USPQ2d at 1406. The test is whether one skilled in the art can recognize the identity of the purportedly described. *Id.*

At oral argument, we were faced with a set of words that the claim alone does not adequately describe. In *Enzo*, 1567, 43 USPQ2d at 1405. In *Enzo*, regardless of whether the claim is an original specification and is not described by the specification as of the date of filing, it is not necessarily met by the specification at 1100 (noting *Eli Lilly's* "original claim" doctrine). In which the name of the claimed invention does not convey sufficient identifying information. If a purported description of an invention does not meet the requirements of the specification, the fact that it appears as an original claim does not save it from being unpatentable. The specification does not save it from becoming more descriptive than, or its longevity.

Under § 112, ¶ 1 requires such disclosure as not persuaded by *Enzo's* argument because the specification indicates "possession" the claimed invention. Inducing three sequences within the claims to practice, *Enzo* needed the invention. It is true that we stated: "The purpose of the 'invention' requirement is broader than to explain how to 'make and use'; must also convey with reason those skilled in the art that, as the invention is sought, he or she was in possession of the invention." *Vas-Cath*, 935 F.2d

at 1563-64, 19 USPQ2d at 1117. That portion of the opinion in *Vas-Cath*, however, merely states a purpose of the written description requirement, viz., to ensure that the applicant had possession of the invention as of the desired filing date. It does not state that possession alone is always sufficient to meet that requirement. Furthermore, in *Lockwood v. American Airlines, Inc.*, we rejected *Lockwood's* argument that "all that is necessary to satisfy the description requirement is to show that one is 'in possession' of the invention." 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Rather, we clarified that the written description requirement is satisfied by the patentee's disclosure of "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention." *Id.*

The articulation of the written description requirement in terms of "possession" is especially meaningful when a patentee is claiming entitlement to an earlier filing date under 35 U.S.C. §§ 119 or 120, in interferences in which the issue is whether a claim is supported by the specification of one or more of the parties, and in *ex parte* applications in which a claim at issue was filed subsequent to the application. See *Vas-Cath*, 935 F.2d at 1560, 19 USPQ2d at 1114 (describing situations in which the written description requirement may arise); *Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985) (noting, in the context of claiming entitlement to the priority date of an earlier application, that the written description requirement is met if "the disclosure of the application relied upon reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter"). Application of the written description requirement, however, is not subsumed by the "possession" inquiry. A showing of "possession" is ancillary to the statutory mandate that "[t]he specification shall contain a written description of the invention," and that requirement is not met if, despite a showing of possession, the specification does not adequately describe the claimed invention. After all, as indicated above, one can show possession of an invention by means of an affidavit or declaration during prosecution, as one does in an interference or when one files an affidavit under 37 C.F.R. § 1.131 to antedate a reference. However, such a

showing of possession alone does not cure the lack of a written description in the specification, as required by statute.

[4] Similarly, we conclude that proof of a reduction to practice, absent an adequate description in the specification of what is reduced to practice, does not serve to describe or identify the invention for purposes of § 112, ¶ 1. As with "possession," proof of a reduction to practice may show priority of invention or allow one to antedate a reference, but it does not by itself provide a written description in the patent specification. We are thus not persuaded by *Enzo's* argument, relying on the PTO's Guidelines, that its disclosure of an actual reduction to practice is an important "safe haven" by which it has demonstrated compliance with the description requirement. The Guidelines state:

Actual reduction to practice may be crucial in the relatively rare instances where the level of knowledge and level of skill are such that those of skill in the art cannot describe a composition structurally, or specify a process of making a composition by naming components and combining steps, in such a way as to distinguish the composition with particularity from all others.

Guidelines, 66 Fed. Reg. at 1101. For biological inventions, for which providing a description in written form is not practicable, one may nevertheless comply with the written description requirement by publicly depositing the biological material, as we have held today. That compliance is grounded on the fact of the deposit and the accession number in the specification, not because a reduction to practice has occurred. Such description is the *quid pro quo* of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time.

CONCLUSION

For the foregoing reasons, we conclude that the district court erred in granting summary judgment that the claims of the '659 patent are invalid for failure to meet the written description requirement of § 112, ¶ 1. While the district judge clearly understood and correctly applied this court's existing precedent, we nevertheless reverse because this case has taken us into new territory and we have held, as a matter of first impression, that reference

in a patent specification to a deposit of genetic material may suffice to describe that material. We therefore remand for further resolution consistent with this opinion.

REVERSED and REMANDED

Enzo Biochem Inc. v. Gen-Probe Inc.

U.S. Court of Appeals
Federal Circuit

No. 01-1230

Decided July 15, 2002

(Non-precedential)

PATENTS

[1] Patentability/Validity — Specification
— Written description (§ 115.1103)

Petition for rehearing is granted, and request for rehearing *en banc* is denied, in action in which appellate panel held that deposit of biological materials in public depository does not necessarily satisfy written description requirement of 35 U.S.C. § 112, and that claims for nucleic acid probes which are described solely in terms of their ability to selectively hybridize to genetic material of bacteria that cause gonorrhea do not satisfy written description requirement.

Appeal from the U.S. District Court for the Southern District of New York, Hellerstein, J.

Action by Enzo Biochem Inc. against Gen-Probe Inc., Chugai Pharma U.S.A. Inc., Chugai Pharmaceutical Co. Ltd., Biomerieux Inc., Becton Dickinson and Co., and Biomerieux SA for patent infringement. Summary judgment of patent invalidity was affirmed on appeal in panel opinion issued April 2, 2002 (62 USPQ2d 1289). On plaintiff-appellant's petition for rehearing, case was referred to merits panel that heard appeal, and panel vacated its decision and prepared revised decision for issuance (63 USPQ2d 1609). Court declined to hear case *en banc*; Lourie, J., joined by Newman, J., concurring in that decision in separate opinion; Newman, J., concurring in separate opinion; Dyk, J., concurring in separate opinion; Rader, J., joined by Gajarsa and Linn, JJ.,

dissenting in separate opinion; Linn, J., joined by Rader and Gajarsa, JJ., dissenting in separate opinion.

[Editor's Note: The U.S. Court of Appeals for the Federal Circuit has indicated that this order is not citable as precedent.]

ON PETITION FOR REHEARING

ORDER

A petition for rehearing was filed by the plaintiff-appellant, and a response thereto was invited by the court and filed by the defendants-appellees. The United States Patent and Trademark Office and Fish & Richardson P.C. filed briefs as *amici curiae*. This matter was referred first to the merits panel that heard this appeal, which vacated its earlier decision and prepared a revised decision for issuance. Thereafter, at the request of a non-panel judge, an *en banc* poll was conducted concerning whether the appeal ought to be heard *en banc*. The poll failed. *Circuit Judges* RADER, GAJARSA, and LINN would have heard the appeal *en banc*.

Upon consideration thereof,

IT IS ORDERED THAT:

[1] The petition for rehearing is granted as set forth in the panel opinion issued concurrently with this order.

LOURIE, *Circuit Judge*, with whom NEWMAN, *Circuit Judge*, joins, filed an opinion concurring in the court's decision not to hear the case *en banc*.

NEWMAN, *Circuit Judge*, filed an opinion concurring in that decision.

DYK, *Circuit Judge*, filed an opinion concurring in that decision.

RADER, *Circuit Judge*, with whom GAJARSA and LINN, *Circuit Judges*, join, filed an opinion dissenting from that decision.

LINN, *Circuit Judge*, with whom RADER and GAJARSA, *Circuit Judges*, join, filed an opinion dissenting from that decision.

Lourie, J., with whom Newman, J., joins, concurring in the court's decision not to hear the case *en banc*.

I agree that the court correctly declined to hear this case *en banc*.

First, it is important to note that the earlier panel majority, in response to the petition for rehearing, has reversed its earlier decision. Taking the case *en banc* would therefore delay and hence frustrate the remand of the case

qualify as "work[s] of visual art" under the statute. See 17 U.S.C. § 101 (defining "work of visual art," in relevant part as "a painting, drawing, print, or sculpture, existing in a single copy, in a limited edition of 200 copies or fewer that are signed and consecutively numbered by the author . . .").

H. LEAVE TO AMEND

[11] Although generally disfavored,¹⁵ the Court has discretion to grant a party leave to amend its complaint even after that complaint is dismissed on summary judgment. In this case, the Court finds reason to do so. First, the Court recognizes that, because of deficiencies in the complaint described above, framing the issues has proved difficult and to a large extent overshadowed resolving those issues. It is conceivable that a properly framed complaint might raise triable issues with regard to some of the claims that pose the difficulties discussed above, although in much more limited scope. Second, the Court notes that, because Abercrombie insisted on proceeding with its motion for summary judgment before it had sought any discovery, discovery in this case was relatively modest. Ordinarily, the type of challenge to the complaint mounted here arises in the context of Rule 12 motions for dismissal for failure to state a claim or for judgment on the pleadings, in which case the Court routinely grants leave to replead unless it determines that any amendment would be futile. Accordingly, the Court shall stay the entry of judgment in this case for 30 days to allow Maharishi to consider whether it is able to remedy the defects with respect only to its claims for trade dress infringement, unfair competition and trade dress dilution, and, if so, to file a motion for leave to amend the complaint. In the event Maharishi fails to file a motion for leave to amend in that time, or otherwise indicates to the Court that it elects not to do so, the Court will issue an amended and final judgment in this case in favor of Abercrombie.

¹⁵ See, e.g., *Freeman v. Continental Gin Co.*, 381 F.2d 459, 469-70 (5th Cir. 1967) ("Much of the value of summary judgment procedure in the cases for which it is appropriate . . . would be dissipated if a party were free to rely on one theory in an attempt to defeat a motion for summary judgment and then, should that theory prove unsound, come back long thereafter and fight on the basis of some other theory.")

IV. ORDER

For the reasons discussed, it is hereby **ORDERED** that the motion of defendants, Abercrombie & Fitch Company and Abercrombie & Fitch Stores, Inc., (collectively, "Abercrombie") for summary judgment dismissing all of the claims of the complaint herein is granted and the complaint is dismissed; it is further

ORDERED that entry of judgment shall be stayed for thirty (30) days from the date of this Order to permit plaintiff Maharishi Hardy Blechman Ltd. ("Maharishi") to file a motion for leave to amend its complaint with respect to its claims for trade dress infringement, unfair competition, and trade dress dilution. In the event Maharishi fails to file such a motion, or otherwise indicates to the Court that it elects not to do so, the Court shall issue an amended judgment in favor of Abercrombie as to all claims.

SO ORDERED.

Noelle v. Lederman

U.S. Court of Appeals
Federal Circuit

No. 02-1187

Decided January 20, 2004

PATENTS

[1] Patentability/Validity — Specification — Written description (§ 115.1103)

Patent claim directed to any antibody which is capable of binding to particular antigen has sufficient support in written description that discloses "fully characterized" antigens; thus, if applicant has disclosed fully characterized antigen, either by structure, formula, chemical name, or physical properties, or by depositing protein in public depository, then applicant can claim antibody by its binding affinity to that described antigen.

[2] Patentability/Validity — Date of invention — In general (§ 115.0401)

Patentability/Validity — Specification — Written description (§ 115.1103)

Applicant's claims to human form of "CD40CR" antibody in continuation applica-

IV. ORDER

or the reasons discussed, it is hereby ORDERED that the motion of defendants, Abercrombie & Fitch Company and Abercrombie & Fitch Stores, Inc., (collectively, "Abercrombie") for summary judgment dismissing all of the claims of the complaint is granted and the complaint is dismissed; it is further

ORDERED that entry of judgment shall be entered for thirty (30) days from the date of Order to permit plaintiff Maharishi Hardiman Ltd. ("Maharishi") to file a motion to amend its complaint with respect to its claims for trade dress infringement, unfair competition, and trade dress dilution. In event Maharishi fails to file such a motion, otherwise indicates to the Court that it does not do so, the Court shall issue an amended judgment in favor of Abercrombie & Fitch.

ORDERED.

He v. Lederman

U.S. Court of Appeals
Federal Circuit
No. 02-1187

Decided January 20, 2004

ENTS

Patentability/Validity — Specification
— Written description (§ 115.1103)

ent claim directed to any antibody which is capable of binding to particular antigen has sufficient support in written description that recites "fully characterized" antigens; thus, applicant has disclosed fully characterized antigens, either by structure, formula, chemical name, or physical properties, or by depositing a sample in public depository, then applicant may claim antibody by its binding affinity to particular antigen.

Patentability/Validity — Date of invention
— In general (§ 115.0401)

Patentability/Validity — Specification —
Written description (§ 115.1103)

Applicant's claims to human form of "CD40CR" antibody in continuation applica-

69 USPQ2d

tion are not supported by written description in prior application, even though earlier application stated that human CD40CR antibody binds to human CD40CR antigen, since prior application, which only described mouse CD40CR antigen, did not disclose "fully characterized" human CD40CR antigen, and since application therefore attempted to define one unknown by its binding affinity to another unknown; moreover, applicant cannot claim genus form of CD40CR antibody from description of mouse CD40CR antigen, since patentee of biotechnological invention cannot necessarily claim genus after describing only limited number of species, in that there may be unpredictability in results obtained from species other than those specifically enumerated.

[3] Practice and procedure in Patent and Trademark Office — Interference — Rules and rules practice (§ 110.1704)

Board of Patent Appeals and Interferences properly applied "two-way" test in finding that there was no interference-in-fact between senior party's patent and junior party's application, since board determined that person of skill in relevant art would have lacked reasonable expectation of success in obtaining senior party's claimed human form of "CD40CR" antibody if provided with junior party's claimed mouse CD40CR antibody and screening techniques cited by junior party; even though board was not required to conduct second prong of test in order to find no interference-in-fact, it nonetheless found that person of skill in art would have lacked reasonable expectation of success in obtaining junior party's mouse CD40CR antibody if provided with senior party's claimed human CD40CR antibody and same screening methods.

[4] Practice and procedure in Patent and Trademark Office — Interference — Rules and rules practice (§ 110.1704)

Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§ 115.0903.03)

Board of Patent Appeals and Interferences correctly found no interference-in-fact between parties' claims to human form of "CD40CR" antibody, since board properly refused to consider methods of antigen isolation

that were found in specification of junior party's prior application, but were not disclosed in junior party's claims, and since, given state of prior art at time of junior party's application, person of ordinary skill in art would not have had reasonable likelihood of success in isolating human CD40CR antibodies from mouse CD40CR antigen and its antibodies disclosed in junior party's application.

Particular patents — Chemical — Human antibody

5,474,771, Lederman, Chess, and Yellin, murine monoclonal antibody (5c8) recognizes a human glycoprotein on the surface of T-lymphocytes, compositions containing same, finding of no interference-in-fact with application no. 08/742,480, in interference no. 104,415, affirmed.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent interference proceeding (no. 104,415) between Randolph J. Noelle (application no. 08/742,480), junior party, and Seth Lederman, Leonard Chess, and Michael J. Yellin (patent no. 5,474,771), senior party. Junior party appeals from finding of no interference-in-fact. Affirmed.

E. Anthony Figg and Glenn E. Karta, of Rothwell, Figg, Ernst & Manbeck, Washington, D.C., for appellant.

James F. Haley Jr., Margaret A. Pierri, Jane T. Gunnison, and Stanley Den-Kua Liang, of Fish & Neave, New York, N.Y.; John P. White, of Cooper & Dunham, New York, for appellees.

Before Cleverger, Bryson, and Gajarsa, circuit judges.

Gajarsa, J.

This is an appeal from an interference proceeding involving the claims of United States Patent Application Serial No. 08/742,480 (the "480 application") and United States Patent No. 5,474,771 (the "771 patent"). Randolph J. Noelle ("Noelle") is the inventor named on the '480 application. Seth Lederman, Leonard Chess, and Michael J. Yellin (collectively "Lederman") are the inventors named on the '771 patent. Noelle appeals the decision of the United States Patent and Trademark Office,

Board of Patent Appeals and Interferences ("Board"), finding no interference-in-fact between the '480 application and the '771 patent and rejecting claims 51, 52, 53, 56, 59, and 60 of the '480 application pursuant to 35 U.S.C. § 102(b) (2000). *Noelle v. Lederman*, Interference No. 104,415 (Bd. Pat. App. & Int. Oct. 19, 2001). Because the decision of the Board is supported by substantial evidence and is not contrary to law, we affirm.

BACKGROUND

A. Antibodies

This case relates to antibodies and their role in the immune response system. A vertebrate's immune system serves to identify and destroy foreign invading organisms and neutralize the toxic molecules they produce. Antibodies, which are proteins also referred to as immunoglobulins ("Ig"), serve to designate foreign particles, broadly referred to as antigens, for destruction by other components of the immune system such as lymphocytes.¹ Lymphocytes, otherwise known as white blood cells, produce antibodies and destroy antigens. T-cells and B-cells are the two types of lymphocytes needed for antibody production. One specific type of T-cell is the helper T-cell. Helper T-cells recognize antigens and then induce B-cells to produce antibodies through a series of events. First the helper T-cell is activated after it recognizes an antigen. Once activated, the helper T-cell activates the B-cell by a combination of binding with the B-cell and secreting signaling molecules. Once the B-cell is activated, it differentiates,² proliferates, and produces antibodies specific to a particular antigen. The antibodies then circulate in the bloodstream and permeate other bodily fluids, where they bind to the antigen, thereby flagging it for destruction.

The present interference involves competing claims to an antibody ("CD40CR antibody") that represses the cell-to-cell signaling interaction between helper T-cells and B-cells.

¹ For additional background on the function of antibodies, as well as methods of isolating antibodies, see *In re Wands*, 858 F.2d 731, 733-34 [8 USPQ2d 1400] (Fed. Cir. 1988) and *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1368-69 [231 USPQ 81] (Fed. Cir. 1986).

² Cell differentiation is the process of modifying a cell's structure and function in order for it to become more specialized and specific to the invading antigen.

CD40CR antigen³ is found on activated, but not resting, helper T-cells. CD40CR antigen acts as a "key" to unlock a protein ("CD40") located on the surface of resting B-cells. Once CD40CR antigen and CD40 bind, the B-cell begins down the pathway to differentiation, proliferation, and antibody production. The CD40CR antibody binds to the CD40CR antigen located on the T-cell surface, thereby inhibiting its ability to bind to the CD40 receptor located on the resting B-cell. B-cells cannot then become activated, thereby preventing the B-cell from producing antibodies. CD40CR antibodies are useful for treating a hyperactive immune system that causes allergic reactions and autoimmune diseases.

B. The Interference

Noelle's '480 application was filed November 1, 1996. The '480 application is a continuation of application Serial No. 08/338,975 ("the '975 application"), filed November 14, 1994, which is in turn a continuation of application Serial No. 07/835,799 ("the '799 application"), filed on February 14, 1992. The claims of Noelle's '480 application are directed to the genus, murine ("mouse"), chimeric ("hybrid"), humanized, and human forms of the CD40CR monoclonal antibody. Noelle also claims the hybridoma⁴ cell lines that produce the CD40CR antibody.

Lederman's '771 issued patent has an effective filing date of November 15, 1991. Lederman's '771 patent describes and claims the human form of CD40CR monoclonal antibody (the "5c8 antibody"). The 5c8 antibody binds to "the 5c8 antigen located on the surface of activated T cells and thereby inhibits T cell activation of B cells." Also, Lederman claims a hybridoma cell line created to produce monoclonal antibody 5c8.

³ CD40CR antigen is also referred to as "CD40 counter receptor," "CD40 ligand," "CD40L," and simply "CD40CR." Lederman uses the term "5c8 antigen" or "T-B cell-activating molecule" ("T-BAM") to designate the 30-kilodalton human form of CD40CR antigen. Noelle uses the term "gp39" (glycoprotein 39 kD) to describe the 39-kilodalton mouse form of CD40CR antigen.

⁴ A hybridoma is a man-made tissue culture consisting of cancerous B-cells fused to B-cells producing the antibody of choice. A hybridoma produces unlimited amounts of a desired "monoclonal" antibody. See *Hybritech*, 802 F.2d at 1368-69 (explaining the method for creating and using hybridomas).

D40CR antigen³ is found on activated, but not resting, helper T-cells. CD40CR antigen acts as a "key" to unlock a protein ("CD40") located on the surface of resting B-cells. Once D40CR antigen and CD40 bind, the B-cell begins down the pathway to differentiation, proliferation, and antibody production. The D40CR antibody binds to the CD40CR antigen located on the T-cell surface, thereby inhibiting its ability to bind to the CD40 receptor located on the resting B-cell. B-cells cannot then become activated, thereby preventing B-cell from producing antibodies. D40CR antibodies are useful for treating a pervasive immune system that causes allergic reactions and autoimmune diseases.

The Interference

Noelle's '480 application was filed November 1, 1996. The '480 application is a continuation of application Serial No. 08/338,975 (the '975 application"), filed November 14, 1994, which is in turn a continuation of application Serial No. 07/835,799 ("the '799 application"), filed on February 14, 1992. The claims of Noelle's '480 application are directed to the genus, murine ("mouse"), chimeric ("hybrid"), humanized, and human forms of the CD40CR monoclonal antibody. Noelle also claims the hybridoma⁴ cell lines that produce the CD40CR antibody.

Lederman's '771 issued patent has an effective filing date of November 15, 1991. Lederman's '771 patent describes and claims the human form of CD40CR monoclonal antibody ("5c8 antibody"). The 5c8 antibody binds to the 5c8 antigen located on the surface of activated T cells and thereby inhibits T cell activation of B cells.⁵ Also, Lederman claims a hybridoma cell line created to produce monoclonal antibody 5c8.

D40CR antigen is also referred to as "CD40 receptor," "CD40 ligand," "CD40L," and simply "CD40CR." Lederman uses the term "5c8 anti-T-B cell-activating molecule" ("T-BAM") to name the 30-kilodalton human form of CD40CR. Noelle uses the term "gp39" (glycoprotein 39) to describe the 39-kilodalton mouse form of CD40CR antigen.

A hybridoma is a man-made tissue culture consisting of cancerous B-cells fused to B-cells producing the antibody of choice. A hybridoma produces unlimited quantities of a desired "monoclonal" antibody. See Hytko, 802 F.2d at 1368-69 (explaining the method for creating and using hybridomas).

On September 3, 1999, an interference was declared by the United States Patent and Trademark Office ("USPTO") between the issued claims of Lederman's '771 patent and Noelle's '480 application. Noelle was designated the junior party and Lederman was designated the senior party based on their effective filing dates. The USPTO established only one count in the interference. The count reads as follows:

The monoclonal antibody of claim 1 of 5,474,771 or the monoclonal antibody of claim 42 or claim 51 of 08/742,480.

Claim 1 of Lederman's '771 patent reads as follows:

A monoclonal antibody, which specifically binds and forms a complex with the 5c8 antigen located on the surface of activated T cells and thereby inhibits T cell activation of B cells, the 5c8 antigen being an antigen to which monoclonal antibody 5c8 (ATCC Accession No. HB 10916) specifically binds.

Claim 42 of Noelle's '480 application reads as follows:

A monoclonal antibody or fragment thereof which specifically binds to an antigen expressed on activated T cells, wherein said antigen is specifically bound by the monoclonal antibody secreted by hybridoma MR1 which hybridoma has been deposited and accorded ATCC Accession No. HB 11048.

Claim 51 of Noelle's '480 application reads as follows:

A monoclonal antibody or fragment thereof which specifically binds CD40CR.

Claim 52 of Noelle's '480 application reads as follows:

The monoclonal antibody or fragment of Claim 51, wherein said CD40CR is expressed by activated human T cells.

For sake of the simplicity, Claim 1 of Lederman's '771 patent and Claim 52 of Noelle's '480 application will be referred to as claims to the "human" form of CD40CR antibody. Claims 42 and 51 of Noelle's '480 application will be referred to as claims to the "mouse" and "genus" forms of CD40CR antibody, respectively.

On June 28, 2001 the Board held a hearing to dispose of the parties' preliminary motions. Lederman moved to have Noelle's claims rejected and sought to redefine the count. Likewise, Noelle also sought to have the count redefined. The Board denied Lederman's motions for judgment against Noelle's mouse claims for lack of written description, lack of enablement, and indefiniteness. See 35 U.S.C. § 112 (2000). The Board found that Lederman had failed to demonstrate that the mouse claims in Noelle's '480 application failed to comply with 35 U.S.C. § 112, paragraphs (1) and (2), as of November 1, 1996, the date Noelle filed his '480 application. The Board, however, determined that the human and genus claims in Noelle's '480 application failed to comply with the written description requirement pursuant to 35 U.S.C. § 112, paragraph (1), as of February 14, 1992, the date Noelle filed the previous '799 application. The Board made a detailed analysis of this court's precedent pertaining to the doctrine of written description, focusing on the holding from *Regents of the University of California v. Eli Lilly & Co.* that an "adequate written description of a DNA sequence claim requires a precise definition, such as structure, formula, chemical name, or physical properties." 119 F.3d 1559, 1566 [43 USPQ2d 1398] (Fed. Cir. 1997). The Board analogized the DNA claims from *Regents* to the antibodies in Noelle's application. Accordingly, the Board held that Noelle's claims regarding the genus and human claims from the '480 application lacked written description support in the specification of Noelle's earlier '799 application because Noelle failed to describe any structural features of the human or genus antibodies or antigens. In other words, the Board found that the claims covering the genus and human antibodies constituted new matter because they lacked adequate written description in Noelle's earlier '799 application. The Board did not reject the claims, but rather denied them the benefit of the earlier filing date of Noelle '799.

Next, the Board addressed the implication of finding a lack of written description for the genus and human claims in Noelle's '480 application. The Board determined that the claims to the human and genus forms of CD40CR antibody in Noelle's '480 application were anticipated by either Lederman '771, which claims priority to U.S. Application 07/

792,728, filed November 15, 1991, or Armitage 5,961,974 (the "974 patent"), which claims priority to U.S. applications 07/783,707 and 07/805,723 filed October 25, 1991, and December 5, 1991, respectively. Noelle had not attempted to distinguish his human and genus claims from the prior art and had conceded that Lederman '771 and Armitage '974 would anticipate those claims if the '480 application were not afforded the earlier filing date of Noelle's '799 application. Thus, the Board found the genus and human claims of Noelle's '480 application to be anticipated under 35 U.S.C. § 102(b) by the two forms of prior art and, as a result, rejected the claims to the human and genus forms of CD40CR antibodies and their respective cell lines pursuant to 37 C.F.R. § 1.641.

On October 19, 2001, the Board ruled on the motions remaining from the previous hearing. The Board had determined in its previous hearing that the deferred motions were essentially requests to decide whether an interference-in-fact existed between the two parties' claims. Lederman then withdrew his pending motions and filed a new motion requesting that the Board find no interference-in-fact.

The Board concluded from the evidence submitted that there was no interference-in-fact. The Board reasoned that a person of ordinary skill in the art lacked a reasonable expectation of success of obtaining the other party's claimed invention given the state of the art at the time. The Board noted three different methods disclosed in Noelle's '480 specification by which a person of ordinary skill in the art could have isolated the human form of the CD40CR antibody given the mouse version of the CD40CR antibody. Dr. Edward A. Clark, Noelle's expert, declared that a person skilled in the art would have had a reasonable expectation of success in isolating human CD40CR antibody by utilizing the methods disclosed in Noelle's specification.

First, Clark testified that human CD40CR antibody could be isolated by immunizing a host with human CD40CR antigen expressing cells or cell lines and selecting the antibody to the CD40CR antigen by functional or competition binding with CD40-Ig.³ Next, Clark suggested methods of making and isolating

antibodies using affinity purified human CD40CR antigen. Last, Dr. Clark declared that one skilled in the art could use the mouse CD40CR antibody or CD40-Ig to clone CD40CR antigen DNA using a method known as expression cloning.

The Board found that one skilled in the art would not have had a reasonable expectation of success of isolating human CD40CR antibodies given the mouse form of CD40CR antigen. At the outset, the Board reasoned that any reference to Noelle's own specification as prior art was improper because the specifications underlying the respective claims cannot be considered "prior art" and an interference-in-fact analysis requires the comparison between the parties' claims, not their specifications. *In re Vaack*, 947 F.2d 488, 493 [20 USPQ2d 1438] (Fed. Cir. 1991). Nevertheless, the Board refused the three methods disclosed in Noelle's specification and endorsed by Clark. First, the Board found that the immunization technique found in the prior art would be ineffective because, at the relevant time, one skilled in the art would not have had a reasonable expectation of success of identifying the activated T-cells that produced the required CD40CR antigen or of isolating the antigen itself. Second, the Board found that it would have been "extremely difficult" for a person of ordinary skill in the art to isolate successfully CD40-Ig, which, as Noelle asserted, could then be used to obtain the claimed CD40CR antibodies. Third, the Board cited statements made during the prosecution of Armitage application 07/969,703 for the proposition that a skilled artisan could not have used expression cloning to isolate CD40CR antibody with a reasonable likelihood of success.

Thus, the Board determined that a person of ordinary skill in the art would not have been reasonably likely to isolate human CD40CR antibody given Noelle's claimed invention of mouse CD40CR antibody. As a result, the Board found no interference-in-fact between Noelle's remaining murine CD40CR antibody claim and Lederman's claim to the human form of CD40CR antibody. Noelle timely appealed to this court and we have jurisdiction under 28 U.S.C. § 1295(a)(4)(A) (2000).

³ CD40-Ig is a fusion protein wherein a portion of the CD40 receptor is fused to an immunoglobulin (Ig). CD40-Ig is therefore not expressed on the surface of a

B-cell but rather is essentially a soluble, free-floating molecule.

antibodies using affinity purified human CD40CR antigen. Last, Dr. Clark declared that one skilled in the art could use the mouse CD40CR antibody or CD40-Ig to clone CD40CR antigen DNA using a method known as expression cloning.

The Board found that one skilled in the art would not have had a reasonable expectation of success of isolating human CD40CR antibodies given the mouse form of CD40CR antigen. At the outset, the Board reasoned that any reference to Noelle's own specification as prior art was improper because the specifications underlying the respective claims cannot be considered "prior art" and an interference-in-fact analysis requires the comparison between the parties' claims, not their specifications. *In re Vaeck*, 947 F.2d 488, 493 [20 USPQ2d 1438] (Fed. Cir. 1991). Nevertheless, the Board refuted the three methods disclosed in Noelle's specification and endorsed by Clark. First, the Board found that the immunization technique found in the prior art would be ineffective because, at the relevant time, one skilled in the art would not have had a reasonable expectation of success of identifying the activated T-cells that produced the required CD40CR antigen or of isolating the antigen itself. Second, the Board found that it would have been "extremely difficult" for a person of ordinary skill in the art to isolate successfully CD40-Ig, which, as Noelle asserted, could then be used to obtain the claimed CD40CR antibodies. Third, the Board cited statements made during the prosecution of Armitage application 07/969,703 for the proposition that a skilled artisan could not have used expression cloning to isolate CD40CR antibody with a reasonable likelihood of success.

Thus, the Board determined that a person of ordinary skill in the art would not have been reasonably likely to isolate human CD40CR antibody given Noelle's claimed invention of mouse CD40CR antibodies. As a result, the Board found no interference-in-fact between Noelle's remaining murine CD40CR antibody claim and Lederman's claim to the human form of CD40CR antibody. Noelle timely appealed to this court and we have jurisdiction under 28 U.S.C. § 1295(a)(4)(A) (2000).

-cell but rather is essentially a soluble, free-floating molecule.

DISCUSSION

Whether a specification complies with the written description requirement of 35 U.S.C. § 112, paragraph (1), is a question of fact. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1562 [19 USPQ2d 1111] (Fed. Cir. 1991), and is, in appeals from the Board, reviewed under the substantial evidence standard. *In re Gartside*, 203 F.3d 1305, 1315 [53 USPQ2d 1769] (Fed. Cir. 2000). To apply a substantial evidence standard, this court must "examin[e] the record as a whole, taking into account evidence that both justifies and detracts from an agency's decision." *Id.* at 1312. A reviewing court must ask "whether a reasonable fact finder could have arrived at the agency's decision." *Id.* "[T]he possibility of drawing two inconsistent conclusions from the evidence does not prevent an administrative agency's finding from being supported by substantial evidence." *Id.*

A. Entitlement to Priority

The written description requirement has been defined many times by this court, but perhaps most clearly in *Vas-Cath*. The court held as follows:

35 U.S.C. § 112, first paragraph, requires a "written description of the invention" which is separate and distinct from the enablement requirement. The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. The invention is, for purposes of the "written description" inquiry, whatever is now claimed.

Vas-Cath, 935 F.2d at 1563-64 (emphasis in original). Thus, the test to determine if an application is to receive the benefit of an earlier filed application is whether a person of ordinary skill in the art would recognize that the applicant possessed what is claimed in the later filed application as of the filing date of the earlier filed application. An earlier application that describes later-claimed genetic material only by a statement of function or result may be insufficient to meet the written description requirement. See *Regents*, 119 F.3d at 1566. This court has held that a description of DNA "requires a precise definition, such as by structure, formula, chemical name, or

physical properties," not a mere wish or plan for obtaining the claimed chemical invention." *Id.* (quoting *Flers v. Revel*, 984 F.2d 1164, 1170 [25 USPQ2d 1601] (Fed. Cir. 1993)). Therefore, this court has held that statements in the specification describing the functional characteristics of a DNA molecule or methods of its isolation do not adequately describe a particular claimed DNA sequence. Instead "an adequate written description of DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself." *Id.* at 1566-67 (quoting *Flers*, 984 F.2d at 1171). It should be noted, however, that this court in *Vas-Cath* warned that each case involving the issue of written description, "must be decided on its own facts. Thus, the precedential value of cases in this area is extremely limited." *Vas-Cath*, 935 F.2d at 1562 (quoting *In re Driscoll*, 562 F.2d 1245, 1250 [195 USPQ 434] (C.C.P.A. 1977)).

Indeed, the court in *Enzo Biochem v. Gen-Probe, Inc.*, 323 F.3d 956, 964 (Fed. Cir. 2002) ("*Enzo Biochem II*"), stated that "the written description requirement would be met for all of the claims [of the patent at issue] if the functional characteristic of [the claimed invention was] coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed." Also, the court held that one might comply with the written description requirement by depositing the biological material with a public depository such as the American Type Culture Collection ("ATCC"). *Id.* at 970. The court proffered an example of an invention successfully described by its functional characteristics. The court stated:

For example, the PTO would find compliance with 112, paragraph 1, for a claim to an isolated antibody capable of binding to antigen X, notwithstanding the functional definition of the antibody, in light of the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature.

Id. The court adopted the USPTO Guidelines as persuasive authority for the proposition that a claim directed to "any antibody which is capable of binding to antigen X" would have sufficient support in a written description that

disclosed "fully characterized antigens." Synopsis of Application of Written Description Guidelines, at 60, available at <http://www.uspto.gov/web/menu/written.pdf> (last visited Jan. 16, 2003) (emphasis added).

[1] Therefore, based on our past precedent, as long as an applicant has disclosed a "fully characterized antigen," either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository, the applicant can then claim an antibody by its binding affinity to that described antigen.

[2] Noelle did not provide sufficient support for the claims to the human CD40CR antibody in his '480 application because Noelle failed to disclose the structural elements of human CD40CR antibody or antigen in his earlier '799 application. Noelle argues that because antibodies are defined by their binding affinity to antigens, not their physical structure, he sufficiently described human CD40CR antibody by stating that it binds to human CD40CR antigen. Noelle cites *Enzo Biochem II* for this proposition. This argument fails, however, because Noelle did not sufficiently describe the human CD40CR antigen at the time of the filing of the '799 patent application. In fact, Noelle only described the mouse antigen when he claimed the mouse, human, and genus forms of CD40CR antibodies by citing to the ATCC number of the hybridoma secreting the mouse CD40CR antibody. If Noelle had sufficiently described the human form of CD40CR antigen, he could have claimed its antibody by simply stating its binding affinity for the "fully characterized" antigen. Noelle did not describe human CD40CR antigen. Therefore, Noelle attempted to define an unknown by its binding affinity to another unknown. As a result, Noelle's claims to human forms of CD40CR antibody found in his '480 application cannot gain the benefit of the earlier filing date of his '799 patent application.

Moreover, Noelle cannot claim the genus form of CD40CR antibody by simply describing mouse CD40CR antigen. Noelle cites *Staehelein v. Secher*, 24 U.S.P.Q.2d 1513, 1519 (Bd. Pat. App. & Int. Sept. 28, 1992), as support for his argument that he has rights to the genus form of CD40CR antibody. In *Staehelein*, Dr. Secher had developed a hybridoma that produced a monoclonal antibody targeted to an antigen unavailable in pure form. *Id.* The

antigen was human leukocyte interferon. *Id.* In Secher's foreign application, he had reported the isolation of a hybridoma-secreting antibody to human leukocyte interferon. *Id.* In his subsequent U.S. application, Secher claimed the genus form of the antibody. *Id.* at 1520. The Board held, "Secher's disclosure . . . would have reasonably conveyed to a person possessing ordinary skill in the art that Secher possessed the genus later claimed by them in their U.S. application in the sense of 35 U.S.C. 112, first paragraph." *Id.* The Board held it is not necessary to describe the exact details for preparing every species within the genus in order to claim the genus. *Id.* (citing *Utter v. Hiraga*, 845 F.2d 993, 998 [6 USPQ2d 1709] (Fed. Cir. 1988)). Thus, Noelle argues, the disclosure in his previous '799 application of the mouse form of CD40CR antibody was sufficient to support his later genus claims.

Noelle's reliance on *Staehelein* is misplaced. First, it is a decision from the Board of Patent Appeals and Interferences which may be persuasive but it is not binding precedent on this court. Second, the Board in *Staehelein* cited *Utter* to support the proposition that a patentee need not cite every species of an antibody in order to claim the genus of that antibody. In *Utter*, this court held that not every species of scroll compressor used in air conditioners must be described in order for a genus claim to meet the written description requirement. 845 F.2d at 994. Since the Board's decision in *Staehelein*, this court has subsequently held that a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from species other than those specifically enumerated. See *Enzo Biochem II*, 323 F.3d at 965; *Regents*, 119 F.3d at 1568. Therefore, to the extent the Board's decision in *Staehelein* conflicts with our decisions in *Enzo Biochem II* and *Regents*, it has been limited in applicability.

The Board was also correct in its determination that the human and genus claims were anticipated by Lederman '771 and Armitage '974. The Board's decision was supported by substantial evidence, and Noelle conceded that without the earlier filing date of his '799 application, his claims were indistinguishable from the prior art cited by the Board.

antigen was human leukocyte interferon. *Id.* in Secher's foreign application, he had reported the isolation of a hybridoma-secreting antibody to human leukocyte interferon. *Id.* In his subsequent U.S. application, Secher claimed the genus form of the antibody. *Id.* at 1520. The Board held, "Secher's disclosure... would have reasonably conveyed to a person possessing ordinary skill in the art that Secher possessed the genus later claimed by them in their U.S. application in the sense of 35 U.S.C. 112, first paragraph." *Id.* The Board held it is not necessary to describe the exact details for preparing every species within the genus in order to claim the genus. *Id.* (citing *Utter v. Hiraga*, 845 F.2d 993, 998 [6 USPQ2d 1709] (Fed. Cir. 1988)). Thus, Noelle argues, the disclosure in his previous '799 application of the mouse form of CD40CR antibody was sufficient to support his later genus claims.

Noelle's reliance on *Staelin* is misplaced. First, it is a decision from the Board of Patent Appeals and Interferences which may be persuasive but it is not binding precedent on this court. Second, the Board in *Staelin* cited *Utter* to support the proposition that a patentee need not cite every species of an antibody in order to claim the genus of that antibody. In *Utter*, this court held that not every species of scroll compressor used in air conditioners must be described in order for a genus claim to meet the written description requirement. 845 F.2d at 994. Since the Board's decision in *Staelin*, this court has subsequently held that a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from species other than those specifically enumerated. See *Enzo Biochem II*, 323 F.3d at 965; *Regents*, 119 F.3d at 1568. Therefore, to the extent the Board's decision in *Staelin* conflicts with our decisions in *Enzo Biochem II* and *Regents*, it has been limited in applicability.

The Board was also correct in its determination that the human and genus claims were anticipated by Lederman '771 and Armitage '974. The Board's decision was supported by substantial evidence, and Noelle conceded that without the earlier filing date of his '799 application, his claims were indistinguishable from the prior art cited by the Board.

B. Interference-In-Fact

Interference proceedings are subjected to the requirements of 37 C.F.R. §§ 1.601 - 1.690 (2003), promulgated pursuant to 35 U.S.C. § 135(a). *Eli Lilly v. Bd. of Regents of the Univ. of Wash.*, 334 F.3d 1264, 1267 [67 USPQ2d 1161] (Fed. Cir. 2003). A patent interference is designed to "determine whether two patent applications (or a patent application and an issued patent) are drawn to the same 'patentable invention' and, if so, which of the competing parties was first to invent the duplicative subject matter." *Id.* (citing *Conservolite, Inc. v. Widmayer*, 21 F.3d 1098, 1100-01 [30 USPQ2d 1626] (Fed. Cir. 1994)); see also 37 C.F.R. § 1.601(j).⁶ In order to determine whether the two parties claimed the same patentable invention, the USPTO has promulgated a "two-way" test, which has been approved by this court. *Eli Lilly*, 334 F.3d at 1270. The two-way test reads as follows:

Invention "A" is the same patentable invention as an invention "B" when invention "A" is the same as (35 U.S.C. 102) or is obvious (35 U.S.C. 103) in view of invention "B" assuming invention "B" is prior art with respect to invention "A". Invention "A" is a separate patentable invention with respect to invention "B" when invention "A" is new (35 U.S.C. 102) and non-obvious (35 U.S.C. 103) in view of invention "B" assuming invention "B" is prior art with respect to invention "A".

37 C.F.R. § 1.601(n). In order for an interference-in-fact to exist, invention A must anticipate or make obvious invention B, and invention B must anticipate or make obvious invention A, thereby meeting both prongs of the "two-way" test. *Eli Lilly*, 334 F.3d at 1268; accord *Winter v. Fujita*, 53 USPQ2d 1234, 1243 (Bd. Pat. App. & Int. Nov. 16, 1999). The Board in the present case worded the two-way test in a different way as follows:

Thus, for Lederman to succeed in its motion for no interference-in-fact, Lederman need only demonstrate that: (i) Lederman's claims are not anticipated or rendered obvi-

ous by Noelle's remaining "mouse" claims; or (ii) Noelle's remaining "mouse" claims are not anticipated or rendered obvious by Lederman's claims.

(Emphasis in original).

[3] Noelle's argument that the Board improperly required a two-way patentability test, or, as the Board phrased it, a "one-way distinctiveness" test, is without merit in light of this court's recent ruling in *Eli Lilly* upholding the Director's two-way test as consistent with the language of the regulation. 334 F.3d at 1268. Therefore, the Board applied the proper "two-way test." First, it determined that "one skilled in the art lacked a reasonable expectation of success of obtaining Lederman's claimed 'human' subject matter when provided with Noelle's 'mouse' subject matter and using the screening techniques cited by Noelle." Although the Board did not have to conduct the second prong of the test to find no interference-in-fact, it did so anyway by finding that "one skilled in the art would have lacked a reasonable expectation of success of obtaining Noelle's 'mouse' subject matter when provided with Lederman's claimed 'human' subject matter and using the same screening methods." Therefore, the Board utilized the correct test to find no interference-in-fact.

Noelle's argument that the Board erred in its application of the obviousness question in the interference-in-fact analysis by ignoring the specification in Noelle's '480 application is also without merit. Both Lederman and Noelle concede that the anticipation portion of the interference-in-fact analysis is not an issue in light of the agreed variance between claims to mouse versus human forms of CD40CR antibodies. Thus, only the obviousness analysis pursuant to 35 U.S.C. § 103 is left to be determined. Obviousness is determined as follows:

a proper analysis under § 103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success.

In re Vaack, 947 F.2d at 493. Both the suggestion and the reasonable expectation of success "must be founded in the prior art, not in the

⁶ 37 C.F.R. § 1.601 (j) reads as follows:

An interference-in-fact exists when at least one claim of a party that is designated to correspond to a count and at least one claim of an opponent that is designated to correspond to the count define the same patentable invention.

applicant's disclosure." *Id.*; see also *In re Dow Chem. Co.*, 837 F.2d 469, 473 [5 USPQ2d 1529] (Fed. Cir. 1988).

The parties agree that a skilled artisan would have been motivated to obtain the human CD40CR antibody if the mouse CD40CR antibody were available. The two parties disagree, however, as to whether the prior art would provide a reasonable likelihood of success in so doing. Therefore, the issue before us is whether substantial evidence supports the Board's determination that one of ordinary skill in the art would not have had a reasonable expectation of success of isolating the other party's invention given the disclosures found in the claims. A reasonable likelihood of success does not necessarily mean an absolute predictability, but rather a reasonable expectation of success. *Yamanouchi Pharm. v. Danbury Pharmacal, Inc.*, 231 F.3d 1339, 1343 [56 USPQ2d 1641] (Fed. Cir. 2000).

Noelle argues that the methods disclosed in his '799 patent application would have provided a reasonable likelihood of success for a person of ordinary skill in the art to isolate human CD40CR antibodies using mouse CD40CR antibodies. Specifically, Noelle argues it would have been obvious to a skilled artisan to use the CD40-Ig fusion protein disclosed in the '799 application as a screen to locate, within a hybridomal library, monoclonal antibodies that specifically bind to human CD40CR antigen. Noelle further argues the Board improperly ignored this method of antibody isolation merely because it was disclosed in Noelle's written description as opposed to Noelle's claims.

[4] The Board correctly found no interference-in-fact between Noelle's claims and Lederman's claims. First, the Board was correct in not considering Noelle's methods of isolation of human CD40CR antigen using CD40-Ig found in his '799 specification because the methods were neither part of the parties' inventions nor "prior art." USPTO rules establish that an interference-in-fact exists when both parties claim the "same patentable invention." 37 C.F.R. § 1.601(n). A patentee's invention is only found in a patentee's claims, unless the patentee uses sufficient means-plus-function language to invoke 35 U.S.C. § 112, paragraph (6). Thus, if the Board is to compare two inventions, the Board must only compare the parties' claims. Noelle does not claim a method of isolating CD40CR

antigens, CD40-Ig, or the receptor CD40 itself. Obviously, if certain terms in Noelle's or Lederman's claims were ambiguous, we could resort to the specification or other sources to define those terms; however, it is unnecessary here as none of the terms in the claims are ambiguous. Therefore, Noelle cannot rely on a method of isolating human CD40CR antigen using CD40-Ig in order to prove obviousness between his invention and Lederman's invention because the method is not claimed.

Second, the Board's determination was supported by substantial evidence because a person of ordinary skill in the art, given the state of prior art at the time of the '799 filing, would not have had a reasonable likelihood of success in isolating human CD40CR antibodies from the mouse CD40CR antigen and its antibody. Noelle argues that one skilled in the art would have had a reasonable likelihood of success in manufacturing a set of hybridomas that secrete monoclonal antibodies to activated human helper T-cell surface antigens. Noelle, as outlined previously, cited three different screening methods disclosed in his specification that would isolate the desired hybridomas and their antibodies. The first two of Noelle's proposed screening methods require the use of CD40Ig. As the expert testimony of Dr. Aruffo, the named inventor in the patent claiming CD40-Ig, indicated to the Board, it would have been unpredictable and unreasonable to expect a skilled artisan to produce CD40-Ig given the state of the art at the time.

Finally, Noelle's expert witness, Dr. Clark, addressed the third and final proposed screening method. Dr. Clark declared that, given the mouse form of CD40CR antibody or CD40-Ig and the utilization of expression cloning methods available at the time, a person of ordinary skill in the art would have had a reasonable expectation of success in isolating the human form of CD40CR antigen. Armitage, however, during the prosecution of his '703 application, stated that the use of expression cloning could not have reasonably led to successful isolation of human CD40CR antigen.

After examining the record as a whole, we conclude there was substantial evidence to support the Board's decision. The Board's decision was reasonable in that, given the state of the art in the early 1990s as described by the expert witnesses, a person of ordinary skill in the art would not have had a reasonable likelihood of success in isolating human

erman

69 USPQ2d

togens, CD40-Ig, or the receptor CD40 itself. Obviously, if certain terms in Noelle's or Lederman's claims were ambiguous, we could sort to the specification or other sources to define those terms; however, it is unnecessary here as none of the terms in the claims are ambiguous. Therefore, Noelle cannot rely on a claim of isolating human CD40CR antigen using CD40-Ig in order to prove obviousness between his invention and Lederman's invention because the method is not claimed.

Second, the Board's determination was supported by substantial evidence because a person of ordinary skill in the art, given the state of prior art at the time of the '799 filing, would not have had a reasonable likelihood of success in isolating human CD40CR antibodies from the mouse CD40CR antigen and its antibody. Noelle argues that one skilled in the art could have had a reasonable likelihood of success in manufacturing a set of hybridomas to secrete monoclonal antibodies to isolated human helper T-cell surface antigens. Noelle, as outlined previously, cited three different screening methods disclosed in his specification that would isolate the desired hybridomas and their antibodies. The first two of Noelle's proposed screening methods require the use of CD40Ig. As the expert testimony of Dr. Aruffo, the named inventor in the patent claiming CD40-Ig, indicated to the Board, it would have been unpredictable and unreasonable to expect a skilled artisan to produce CD40-Ig given the state of the art at the time. Finally, Noelle's expert witness, Dr. Clark, dressed the third and final proposed screening method. Dr. Clark declared that, given the use form of CD40CR antibody or CD40-Ig and the utilization of expression cloning methods available at the time, a person of ordinary skill in the art would have had a reasonable expectation of success in isolating the human form of CD40CR antigen. Armitage, however, rejected the prosecution of his '703 application, stating that the use of expression cloning could not have reasonably led to successful isolation of human CD40CR antigen.

After examining the record as a whole, we conclude there was substantial evidence to support the Board's decision. The Board's decision was reasonable in that, given the state of the art in the early 1990s as described by expert witnesses, a person of ordinary skill in the art would not have had a reasonable likelihood of success in isolating human

69 USPQ2d

King v. Continental Western Insurance Co.

1517

CD40CR antigen given mouse CD40CR antigen.

CONCLUSION

For the foregoing reasons, the decision of the Board rejecting claims 51, 52, 53, 56, 59, and 60 of Noelle's U.S. Application No. 08/742,480 is affirmed. The decision of the Board granting Lederman's preliminary motion of no interference-in-fact is also affirmed.

AFFIRMED

No costs.

King v. Continental Western Insurance Co.

Missouri Court of Appeals

Western District

No. WD 61555

Decided October 28, 2003

COPYRIGHTS

[I] Rights in copyright; infringement — In general (§ 213.01)

Copyright infringement resulting from insured homebuilder's construction of house based on copyrighted plans, without copyright owner's permission, constituted "advertising injury" caused by "offense committed in the course of advertising" homebuilder's goods and services that obligated defendant insurance company, pursuant to terms of commercial general liability policy, to defend homebuilder in infringement action brought by copyright owner, since builder's placement of sign bearing its name outside home site during construction constituted advertising, in that sign was designed to garner business and directed its message at public, since home construction was necessary part of that advertising, and policy does not limit coverage to cases in which offense predominantly functions as advertising, and since copyright owner's failure to explicitly seek damages stemming from home's role as advertising did not obviate coverage.

Appeal from the Missouri Circuit Court, Cole County, Kinder, J.

Action by Kirk King, King Construction Inc., and American Family Mutual Insurance Co. against Continental Western Insurance Co. for breach of contract and vexatious refusal to provide insurance coverage in connection with copyright infringement action brought by third party against plaintiffs Kirk King and King Construction Inc. Defendant appeals from trial court's judgment that defendant had duty to defend plaintiffs in copyright suit. Affirmed.

Mark A. Ludwig, of Carson & Coil, Jefferson City, Mo., for plaintiff-respondent.

Brian K. Francka, of Schreimann, Rackers, Francka & Blunt, Jefferson City, for defendant-appellant.

Before Ellis, chief judge, and Lowenstein and Holliger, judges.

Lowenstein, J.

Continental Western Insurance Company appeals from the judgment of the trial court holding that Continental had a duty to defend Kirk King and King Construction, Inc. (collectively "King"), in a copyright-infringement suit filed against the two. Continental insured King under a commercial general liability policy. Plaintiff-Respondent King, a custom homebuilder was sued for using a copyrighted house plan as its own. The Continental policy covers "advertising injuries" caused by an offense (infringement of copyright) committed in the course of advertising the insured's products. This case pivots on whether the placing of a sign bearing the builder's name next to a construction site of the builder constitutes advertising within the meaning of the insurance policy. Continental argues that the trial court erred in concluding that it had a duty to defend and to indemnify King and that American Family Mutual Insurance Company (American Family), the predecessor insurer of King, must split the defense and indemnification costs. This court rejects both points.

I. Facts

William Wiley sued Kirk King and King Construction, Inc. (and other parties not important to this appeal) for copyright infringement in violation of 17 U.S.C. §§ 101 et seq. Wiley alleged that King had "knowingly, intentionally and willfully copied Wiley's



Tech Resources

- Data Sheet
- MSDS - English
- Other Languages
- Certificate of Analysis

Store

- ★ 2 to 8°C

- Ship

- Blue Ice

Note: Store and Ship conditions may differ.

See Key

Be the first to
know about our



Home

Products

Technical
Resources

Literature

Ordering

Contact Us



Search Help



Cat. No. PC09

Anti-PTHLP (Ab-2) (34-53) Rabbit pAb

All Categories » Calbiochem » Antibodies » Primary Antibodies » Other

Anti-PTHLP

Anti-Parathyroid Hormone-Like Protein

Anti-Parathyroid Hormone-Related Protein

Anti-PTHrP

Host: Rabbit

Isotype: IgG

Immunogen: a synthetic peptide (AEIRATSEVSPNSKPSNTK) corresponding to amino acids 34-53 of human PTHLP

Form: Liquid

Formulation: In 0.05 M sodium phosphate buffer, 0.2% gelatin.

Preservative: ≤0.1% sodium azide

Positive Control: Any renal carcinoma cell line or normal skin

Comments: Recognizes parathyroid hormone-like protein. Does not cross-react with human PTH.

Ref.: Thonkay, M., et al. 1989. *Endocrinology* 124, 111. Danks, J.A., et al. 1988. *J. Bone Min. Res.* 3, S214. Mangin, M., et al. 1988. *Proc. Natl. Acad. Sci. USA* 85, 597. Burtis, W.J., et al. 1987. *J. Biol. Chem.* 262, 7151. Moseley, J.M., et al. 1987. *Proc. Natl. Acad. Sci. USA* 84, 5048. Strewler, G.J., et al. 1987. *J. Clin. Invest.* 80, 1803. Suva, L.J., et al. 1987. *Science* 237, 893. Goddard, J.W., et al. 1986. *Recent Progress Hormone Res.* 42, 705.

Need additional information about this product? Email our Technical Service department at: technical@calbiochem.com

▶ Related information for this product is available:
Additional information is available from the "Tech Resources"
box in the upper left panel of this page.

- Select One -



EMD Chemicals Inc. USD list price is displayed (pricing with local distributors may vary). NOTE: In Stock status is based on item availability worldwide.

[Sales Office Contact Details](#)

Size	In Stock	Qty	Price
100 µg	Y		\$265.00

Add to Cart

Clone	Species Reactivity	Application
	human	FC, IB, IF, IP, PS See Key

Related Products:

GF08: Anti-PTHLP (Ab-1) Mouse mAb (212:10.7)

PC09: Anti-PTHLP (Ab-2) (34-53) Rabbit pAb

Material Safety Data Sheets:

PC09: Anti-PTHLP (Ab-2) (34-53) Rabbit pAb - English

Bulgarian:

Danish:

Dutch:

English:

Spanish:

Swedish:

German:

Greek:

Hungarian:

Italian:

Korean:

Norwegian:

Polish:

Portuguese:

Spanish:

Swedish:

Other:

Related Categories:

[All Categories](#) » [Calbiochem](#) » [Antibodies](#) » [Primary Antibodies](#) » [Other](#)

[Change Country](#) | [Contact Us](#) | [Privacy Policy](#) | [Terms and Conditions](#)

©2009 Calbiochem, Novabiochem, & Novagen
are brands of EMD Chemicals Inc., an Affiliate of Merck KGaA, Darmstadt, Germany.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

MASSFELDER et al.Atty. Ref.: **3665-133**Serial No. **10/520,085**Group: **1643**Filed: **January 5, 2005**Examiner: **Gussow**For: **USE OF PTHRP ANTAGONISTS FOR TREATING RENAL
CELL CARCINOMA**
* * * * *

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

RULE 132 DECLARATION

I, Mustapha OULAD ABDELGHANI, do hereby declare and say as follows:

1. I have reviewed the above-identified application. I have also considered the documents listed herein.

2. I am currently a Research Engineer (IR1, INSERM), in charge of the monoclonal antibody facility at the IGBMC (ILLKIRCH, FRANCE). I hold a Ph D from University of Burgundy, DIJON, FRANCE, earned in (1991).

3. I have been advised by the applicants' French representative that the U.S. Patent Office official in charge of the examination of the application has asserted that the specification allegedly fails to describe the use of an anti-PTHrP (34-53) antibody. I have been advised by the applicants' French representative that the U.S. Patent Office official in charge of the examination of the application has asserted that the specification only describes the use of the Ab-2 anti-PTHrP (34-53) antibody which was

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

available from Oncogene and was used in the specification to exemplify the disclosure of the application.

4. For the reasons detailed herein, I believe that one of ordinary skill in the art will appreciate that the applicants described the general use of an anti-PTHrP (34-53) antibody as an antagonist for treating a kidney cancer and were in possession of such a method as evidenced by the disclosure of the above-identified specification.

5. One of ordinary skill will appreciate that an "anti-PTHrP (34-53) antibody" refers to an antibody that binds residues 34-53 of PTHrP. Evidence of such terminology may be found, for example, in the following:

(1) Okada et al "Immunohistochemical Localization of Parathyroid Hormone-related Protein in Canine Mammary Tumors" Vet Pathol 34: 356-359 (1997) (describing an antibody to "PTHrP (1-36)" (page 358 right column), the use of a "commercially available rabbit-derived anti-PTHrP (34-53) antibody" (id.), and the N-terminus (1-36) and midregion (36-111) of PTHrP);

(2) Verheijen et al, "Parathyroid hormone-related peptide (PTHrP) induces parietal endoderm formation exclusively via the Type I PTH/PTHrP receptor" Mechanisms of Development 81 (1999) 151-161 (describing the N-terminus of PTHrP as "PTHrP (1-34)" (see page 151, left column), the use of the N-terminal fragment "PTHrP(1-34)" and full length version "PTHrP(1-141)" (see page 152, right column), and fragments spanning amino acids 67-86, 87-94 and 107-139 as "PTHrP(67-86)", PTHrP(67-94)" and "PTHrP(107-139)", respectively (see page 153, left column and Figure 1, and the "Materials" section on page 158 which describes the source of peptides and antibodies));

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

(3) Thorikay et al., "Synthesis of a gene encoding parathyroid hormone-like protein-(1-141): purification and biological characterization of the expressed protein" *Endocrinology*, Vol 124, 111-118 (1989) (abstract) (describing "PTHLP" as a 141 amino acid protein designated "PTHLP-(1-141)");

(4) Fenton et al., "A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts," *Endocrinology*, 1991 Oct;129(4):1782-8 (Abstract) (describing a carboxy fragment of PTHrP as "PTHrP-(107-139)");

(5) Santos et al "Up-regulation of parathyroid hormone-related protein in follicle acid-induced acute renal failure" *Kidney International*, vol. 60 (2001), pp 982-995 (describing "anti-PTHrP antibody Ab-2 (Oncogene, Uniondale, NY, USA), [as] recognizing the sequence 34 to 53 of human and rat PTHrP" on page 983);

(6) Garcia-Ocana et al "Cyclosporine increases renal parathyroid hormone-related protein expression in vivo in the rat" *transplantation*, vol 65, 860-863, No. 6, March 27, 1998 (describing "anti-PTHrP antibody Ab-2 (Oncogene, Uniondale, NY), [as] recognizing the sequence (34-53) of human and rat PTHrP" on page 861); and

(7) Richard, et al. "Humoral Hypercalcemia of Malignancy, Severe Combined Immunodeficient/Beige Mouse Model of Adult T-Cell Lymphoma Independent of Human T-Cell Lymphotropic Virus Type-1 Tax Expression" *Am J Pathol.* 2001 June; 158(6): 2219-2228 (describing "polyclonal rabbit anti-PTHrP (PTHrP amino acids 34 to 53) (1:100, Ab-2, Oncogene Research Products, Cambridge, MA)");

Each of the above-noted references describes fragments of PTHrP by the amino acid positions, in parentheses, as recited in the above-identified application.

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

The following references, for example, make similar reference to fragments of PTHrP:

(A) Burton et al., "Parathyroid hormone related peptide can function as an autocrine growth factor in human renal cell carcinoma" 1990, Biochemical and Biophysical Research Communications, Vol. 167, No. 3, pages 1134-1138;

(B) Ogata et al (EP1197225);

(C) Hoare et al "Specificity and stability of a new PTH1 receptor antagonist, mouse TIP(7-39)" Peptides, 2002, vol 23, No. 5, pp 989-998; and

(D) Sato et al (U.S. Patent No. 6,903,194).

6. Sato et al describes "Humanized anti-PTHrP (1-34) Antibody" in Figures 13 and 14. Moreover, Sato et al describes the use of a fragment "[PTHrP(1-34)]" as an antigen to produce antibodies as follows:

"PTHrP used for the immunization of animals includes peptides having the whole or part of the amino acid sequence of PTHrP prepared by recombinant DNA technology or chemical synthesis, and PTHrP derived from supernatants of cancer cells causing hypercalcemia. For example, a peptide [PTHrP(1-34)] comprising the 1st to 34th amino acids of the known PTHrP (Kemp, B. E. et al., Science (1987) 238, 1568-1570) may be used as the antigen." See column 7, lines 48-55 of Sato.

Further, Sato describes antibodies binding human PTHrP as "anti-human PTHrP antibodies" and generally antibodies which bind PTHrP as "Anti-PTHrP Antibody". See column 10, last line, column 22, line 56, and, for example, column 23, lines 25 and 37-38 of Sato.

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

7. One of ordinary skill in the art will appreciate that "an anti-PTHrP (34-53) antibody" is a general recitation of an antibody which binds to the fragment of PTHrP spanning amino acids 34-53.

8. The above-identified specification describes the following as examples of an anti-PTHrP antibody which may be an antagonist according to the disclosure: the anti-PTHrP(1-34) antibodies (human, rat) of Bachem (Bachem Biochimie Sarl, Volsins-le-Bretonneux, France), the anti-PTHrP(34-53) antibody (Ab-2, human) of Oncogene (France Biochem, Meudon, France), the antibody #23-57-137-1 (described in particular in the patent application EP1187225) and the anti-PTHrP(107-139) antibody (human) obtained by conventional methods of antibody preparation. See page 9, lines 10-15 of the above-identified specification. One of ordinary skill in the art will further appreciate from, for example, page 21, line 31 ("anti-PTHrP (34-53)"), page 17, lines 25-28 ("The anti-PTHrP(34-53) antibody (Ab-2, human) was obtained from Oncogene (France Biochem, Meudon France) and the anti-PTHrP(107-139) antibody (human) was a gift of Dr. P. Esbrit (Fundacion, Jimenez Diaz, Madrid, Spain)") and page 27, line 1 ("Int. region: anti-PTHrP (34-53) antibody (Ab-2, Oncogene) 2 µg/ml") of the specification, that the above-identified specification describes the use of anti-PTHrP antibodies from a number of sources as exemplifications of anti-PTHrP antibodies which bind to the amino acid fragment described numerically in parentheses (i.e., fragments of PTHrP spanning amino acids 34-53 and 107-139 in the above-noted passages).

9. The above-identified specification describes in the background section the previous experiences in the art with anti-PTHrP antibodies and that the above-identified application provides a therapy based on the use of PTHrP antagonists to treat patients

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

affected by clear cell carcinoma (CCC). See page 5, last paragraph of the specification. PTHrP antagonists are described in specification as including compounds which decrease the biological effect or effects of PTHrP and can include a compound binding the PTHrP receptor which partially or wholly inhibits binding of PTHrP to its receptor. These antagonists are described as including peptides of PTH or PTHrP comprising a substitution or deletion of at least one amino acid of the sequence of the PTH and or the PTHrP, or a partial sequence of the PTH or PTHrP peptides, optionally comprising a substitution or a deletion of at least one amino acid of their sequence. See page 7, lines 3-14 of the specification.

10. The above-identified specification further describes that specific examples of antagonist compounds binding the PTHrP receptors according to the invention include PTHrP (3-34), PTHrP (7-34), PTHrP (8-34), PTHrP (9-34), PTHrP (10-34), the amides or variants thereof. Variants present a replacement, a deletion or an addition of at least one amino acid such as in particular (Asn10, Leu11, D-Trp12) PTHrP (7-34) amide (human or murine). One of ordinary skill in the art will appreciate that "PTHrP(3-34)", for example, is a peptide containing the amino acid sequence of amino acids 3 to 34 of the PTHrP peptide. The specification further describes that among the above-described polypeptides, are also included those which present a deletion, a substitution, an addition or an insertion of at least one amino acid of the peptide sequence of PTH or PTHrP and which have an antagonist activity in respect of PTHrP. The specification further describes a derivative of TIP (tuberoinfundibular peptide) as a PTHrP antagonist, such as truncated peptides of TIP(1-39) (tuberoinfundibular peptide 1-39), in particular TIP(7-39) and its derivatives which have been described as

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

powerful RPTH1 antagonists (Hoare et al, Peptides 23 : 989-998, 2002). See page 7, lines 14-26 of the specification. The specification further describes that the PTHrP antagonist according to the application may be a non-peptidic compound. See page 7, lines 27-29 of the specification.

11. The above-identified specification describes that a PTHrP antagonist according to the description can be a compound binding a ligand of the PTHrP receptor, thereby partially or even totally inhibiting the binding of PTHrP to its receptor. This compound can be selected from anti-PTHrP antibodies and, more preferably, a humanised anti-PTHrP antibody. See page 7, lines 30-33 of the specification.

12. The specification describes that a PTHrP antagonist according to the invention can be a compound increasing the presence of active VHL, thereby decreasing the biological effect or effects of PTHrP, such as a product of the tumour suppressing gene VHL, which can be obtained in particular by gene therapy. The specification further describes that a PTHrP antagonist according to the invention can be a compound reducing the expression of PTHrP. This compound can bind mRNA or the gene of PTHrP, inhibiting, partially or even totally the expression of PTHrP. This compound can be for example an antisense oligonucleotide of PTHrP, a RNAi, a transcription factor repressing the expression of the PTHrP gene or a compound decreasing the stability of the mRNA of PTHrP. See page 8, lines 1-11 of the specification.

13. The specification describes that several kinds of PTHrP antagonists such as defined throughout the specification can be used for the treatment of kidney cancer. The specification describes, for example, a method of treatment including gene therapy

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

and the administration of a PTHrP antagonist such as an antagonist of the PTHrP receptor or an anti-PTHrP antibody. See page 8, lines 12-17 of the specification.

14. Antagonists are further described in the specification as including a compound, such as an anti-PTHrP antibody, which inhibits the binding of a ligand, such as PTHrP, to a PTHrP receptor. Examples of anti-PTHrP antibodies include antibodies such as a humanised antibody, a human antibody, a chimeric antibody, an antibody (such as the antibody #23-57-137-1 (which binds PTHrP(1-35) see Esaki et al "The selection of therapeutic antibodies by kinetic analysis" Biocore Journal – Number 2 2002, pages 7-8)) obtained from a hybridoma (such as the hybridoma #23-57-137-1) or a fragment of an anti-PTHrP which inhibits binding of a ligand to the receptor and/or a modified form of such a fragment. The antibody can be polyclonal or monoclonal. See page 8, lines 23-31 and page 9, lines 4-9 of the specification.

15. The above-identified specification describes the following as examples of an anti-PTHrP antibody which may be an antagonist according to the disclosed invention: the anti-PTHrP(1-34) antibodies (human, rat) of Bachem (Bachem Biochimie Sarl, Voisins-le-Bretonneux, France), the anti-PTHrP(34-53) antibody (Ab-2, human) of Oncogene (France Biochem, Meudon, France), the antibody #23-57-137-1 (described in particular in the patent application EP1197225) and the anti-PTHrP(107-139) antibody (human) obtained by conventional methods of antibody preparation. See page 9, lines 10-15 of the above-identified specification.

16. The specification further describes the use of fragments of PTHrP as antigens to produce anti-PTHrP antibodies in mammals, such as a rodent (e.g., mouse, rat or hamster), a rabbit or a monkey. See page 9, line 29 through page 10, line 4 of

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

the specification. Production of anti-PTHrP antibodies from human cells is also described. See page 11, lines 16-26 of the specification. Recombinant anti-PTHrP antibodies are described. See page 11, line 27 through page 12, line 2 of the specification.

17. Page 14, lines 12-14 of the specification describes the results of Figure 4 of the specification as showing the "effect of the antibodies against the various regions of PTHrP on the proliferation of the tumor cells 786-0 *in vitro* measured by the number of cells...". Figure 4 describes these "regions" as "N-term", "Region int" and "C-term". The specification further defines these regions as follows (see page 26, line 33 through page 27, line 2 of the specification):

"N-term: anti-PTHrP(1-34) antibody (Bachem) 1.5 µg/ml

Int. region: anti-PTHrP (34-53) antibody (Ab-2, Oncogene) 2 µg/ml

C-term: anti-PTHrP(107-139) antibody (P. Esbrit, Madrid, Espagne) 5 µg/ml".

18. One of ordinary skill in the art will appreciate that Figure 4 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants disclosure and description of the invention.

19. Figure 6 of the specification similarly describes results relating to anti-PTHrP antibodies which bind to regions "N-term" (i.e., PTHrP(1-34)), "Region int." (i.e. PTHrP (34-53)), and "C-term" (i.e., PTHrP(107-139)) of PTHrP. The corresponding description of the specification (i.e., page 27, lines 14-23) describes the general applicability of the

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

results of Figure 6 as representing "the effect of the antibodies directed against the different regions of PTHrP on the proliferation of the UOK-128 tumor cells *in vitro* ...".

20. One of ordinary skill in the art will appreciate that Figure 6 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants invention.

21. Figure 8 of the specification similarly describes results relating to anti-PTHrP antibodies which bind to regions "N-term" (i.e., PTHrP(1-34)), "Region Int." (i.e. PTHrP (34-53)), and "C-term" (i.e., PTHrP(107-139)) of PTHrP. The corresponding description of the specification (i.e., page 28, lines 1-12) describes the general applicability of the results of Figure 8 as representing "the effect of the antibodies directed against the different regions of PTHrP on the proliferation of the UOK-128 tumor cells *in vitro* ...".

22. One of ordinary skill in the art will appreciate that Figure 8 of the specification describes results of antibodies binding to regions of PTHrP generally, which are described as being applicable to any anti-PTHrP antibody binding the noted region of PTHrP, and are considered a demonstration of the applicants invention.

23. One of ordinary skill in the art will appreciate, from the whole of the specification, that the applicants were in possession of the claimed invention, relating to the use of an anti-PTHrP (34-53) antibody. The specification is not limited to the specific Ab-2 anti-PTHrP (34-53) antibody used in the examples.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and

MASSFELDER et al.
Application No. 10/520,085
Rule 132 DECLARATION

further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this __27th__ day of __November__, 2008.

(Signature) _____



(print name) __Mustapha OULAD ABDELGHANI__